2-METHYL-2-BUTENE

DOSE RANGE FINDING STUDY IN RATS

BY INHALATION EXPOSURE

ACC Reference Number: OLF-63.0-HVP3-HLS

CSS 001/010176

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Sponsor

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APPENDICES

AUTHOR'S SIGNATURE PAGE

The study described in this report generally followed Good Laboratory Practice principles, however, no specific study related Quality Assurance procedures were performed and the report may not contain all the elements required by GLP.

A retention sample of the test substance was not taken nor held as it was not indefinitely stable and posed a safety hazard.

Amanda Brooker .... 22 April 2004

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Date

This final report was accepted on the behalf of the Sponsor.

.... 1 April 2004

Elizabeth J. Moran,
Sponsor's Representative.

Date

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SUMMARY

This study was performed to assess the effect of the test substance, 2-methyl-2-butene, on pregnant female rats to establish suitable dosages for a 4-week general toxicity and reproductive/developmental toxicity screening study.

Three groups, each of 6 time-mated female Crl:CD® rats, were exposed to an aerosol of 2-methyl-2-butene, 6 hours a day, from Days 12-19 after mating using a whole body exposure system. A fourth group, also of 6 females, acted as a control and was exposed to air only. The doses of 2-methyl-2-butene administered were 2000, 4000 and 7000 ppm for Groups 2, 3 and 4 respectively.

During the study, clinical signs, bodyweight and food consumption were recorded. On Day 20 of pregnancy the animals were killed, examined macroscopically, and the uterus excised for examination of litter parameters.

The following comments in relation to principal findings are made in summary:

Achieved concentration

The chamber mean analysed concentration over the duration of the study were 0 (Control), 1971, 4027 and 7109 ppm in Group 1 to 4 respectively.

Clinical signs and mortality

There were no treatment related clinical observations and no deaths amongst adult females.

Bodyweight

Bodyweight gain in the test animals was transiently lower than controls during the first 4 days of the exposure period, however there was no dosage relationship established.

Food consumption

Food consumption of the test animals was lower than that of the control during the dosing period.

Macroscopic pathology

No treatment related macroscopic changes were noted.

Litter parameters

There were no obvious effects of treatment on the *in-utero* parameters investigated. External necropsy examination of foetuses at Day 20 after mating revealed no abnormalities.

Conclusion

It was concluded that pregnant females would tolerate exposures up to 7000 ppm and that this concentration would be suitable for use in the subsequent OECD 422 study.

INTRODUCTION

The purpose of this study performed at Huntingdon Life Sciences Limited, Huntingdon, England was to assess the influence of the test substance on select aspects of pregnancy following exposure of the rats to repeated whole body inhalation administration of the test substance 2-methyl-2-butene, for 6 hours a day, from Day 12-19 after mating.

The study was performed mainly to determine suitable dosages for a main 4-week general toxicity and reproductive/development screening study.

The study was not designed to meet any specific regulations or guidelines.

The test substance was administered by inhalation, a potential route of human exposure. The rat was the species of choice due to the requirement for a non-rodent species by regulatory agencies. The strain was selected on account of the availability of background data, relating to clinical and pathological parameters, at our laboratories.

RELEVANT STUDY DATES

Approved by:	
Study Director:	19 June 2001
HRC Management:	19 June 2001
Study Sponsor:	12 July 2001
Animals arrived at HRC:	10 August 2001
Exposures commenced:	20 August 2001
Terminal kill:	28 August 2001
Experimental completion date:	28 August 2001

TEST SUBSTANCE

Identity:	2-methyl-2-butene
Alternative identity:	2-methyl, 2-butene
CAS number:	513-35-9
Lot number:	A0153320
Stability:	The test substance was analysed in an investigation conducted as part of HLS Study Number CSS/007 (see Appendix 6 for Certificate of Analysis), which showed that it was stable for the duration of testing in this study.
Purity:	> 98% (Appendix 6)
Appearance:	Clear, colourless liquid
Storage conditions:	In a cool, dry, well-ventilated area
Source:	Fisher Scientific UK Bishop Meadow Road Loughborough Leicester LE11 5RG UK
Date received:	16 May 2001

EXPERIMENTAL PROCEDURE

ANIMAL MANAGEMENT

A total of twenty four sexually mature female rats, approximately 9-10 weeks of age, of the Crl:CD®BR strain, which were time-mated to males of the same strain, were obtained from Charles River (UK) Ltd, Manston Road, Margate, Kent, England. The day of mating was considered Day 0 of pregnancy.

For those animals selected for the study, their estimated age at the start of treatment was 10-11 weeks and their bodyweights were in the range 264 g to 341 g.

The latest Health Screen Report published by the animal supplier was provided to HLS. In addition, the additional consignments of animals included a health screen relating to the current status of the breeding colony. These documents were sent to Huntingdon Life Sciences Veterinary Services immediately upon receipt for review and subsequent archiving.

Animals were assigned to groups randomly on arrival. From each delivery box, in no selective order, animals were allocated to labelled cages, commencing with the first cage, then the second and so on until three animals had been placed in each cage. A review of the mating details provided by the animal supplier confirmed that no females allocated to the same group had been mated with the same male.

The rats were housed in suspended stainless steel cages fitted with wore mesh tops and floors so that each cage contained 3 animals of the same sex. Plastic trays lined with absorbent paper were placed below each cage to collect animal excreta and the paper was changed daily. The rats were kept in a single room and cages from each group were positioned on an individual cage battery. The batteries holding each group of rats were housed in a separate ventilation cabinet within the animal room. For the daily exposures, the animals were transferred to exposure chambers where they were housed in individual animal exposure cages of the suspended basket type, constructed of stainless steel mesh.

Animal room temperature and relative humidity controls were set at 19-25°C and 40-70% respectively. The actual recorded ranges were 21 to 22°C and 48 to 60% for temperature and relative humidity respectively. Permanent weekly recordings of these parameters were made using a Kent Clearspan recorder and these are archived with all other raw data for this study. Artificial lighting was controlled to give 12 hours light (0600 - 1800 hours) and 12 hours dark per 24 hours.

Animals had no access to food or water during each 6-hour exposure. At all other times, all rats had free access to tap water and pelleted UAR VRF1 Certified Diet. There was no information available to the Study Director to indicate that any non-nutrient substance likely to influence the effect of the test compound could reasonably be expected to be present in the diet or the drinking water, both of which were routinely subjected to regular chemical analyses. The results of these analyses are lodged in the Huntingdon Life Sciences Archives.

The animals arrived on Day 1 of gestation. There was an acclimatisation period of 11 days before the start of exposures. The spare animals were retained during this period to replace any rat that showed signs of ill health. These spare rats were discarded once treatment had commenced with no further investigations performed.

ANIMAL IDENTIFICATION

Group	Rat numbers
1 (Air control)	1-6
2 (Low dose)	7-12
3 (Inter dose)	13-18
4 (High dose)	19-24

Each cage was identified by a coloured label according to group, and each label was uniquely numbered with the cage and study number. The animal number was tattooed on the tail of each animal in the cage.

ADMINISTRATION

The test substance, 2-methyl-2-butene, a colourless liquid, was administered to rats by inhalation of a vapour for 6 hours a day using a whole body exposure system (0.75 m^3 exposure chambers). The control animals received air alone.

Group/colour code	Target exposure concentration (ppm)
1: White	0
2: Yellow	2000
3: Blue	4000
4: Pink	7000

The animals were exposed for 8 consecutive days (Days 12 and 19 inclusive of gestation). The animals were exposed at approximately the same time each day and a separate exposure chamber was used for each group.

Details of the exposure system, generation and sampling of the test atmospheres together with the results obtained are presented in the inhalation exposure and analysis section of this report (**ADMINISTRATION OF 2-METHYL-2-BUTENE BY INHALATION TO RATS**).

OBSERVATIONS AND MEASUREMENTS

Dated and signed records of all activities relating to the day-to-day running and maintenance of the study, as well as to the group observations and examinations outlined in this procedure were recorded in the Study Daybook. In addition, observations relating to individual animals made throughout the study were recorded.

Clinical signs and mortality

Individual animals were observed at least twice daily for any signs of behavioural changes, reaction to treatment or ill health. In addition, detailed observations were made daily, on the days of exposure, as follows:

1. Pre exposure observations.
2. Observations during exposure.
3. Observations within ½ to 1 hour of return to home cage

During the daily exposure, obvious signs were recorded as a group response. Due to the type of exposure system used, the ability to observe individual animals during the exposures was severely restricted.

Dated and signed records of appearance, change and disappearance of clinical signs were maintained for individual animals.

Throughout the study, checks were made early in the working day and again in the afternoon to look for dead and moribund animals.

Bodyweight

The weight of each rat was recorded on Days 2, 4, 8, 12, 16 and 20 after mating. During the exposure period, bodyweights were recorded before the daily exposure. All animals were weighed at necropsy.

Food consumption

The quantity of food consumed by each cage of rats was recorded at the following intervals:

Days 2-3, 4-7, 8-11, 12-15 and 16-19 inclusive.

Food intake per rat (g/rat/day) was calculated using the total amount of food given to and left by each cage in each group over each period and the number of rats surviving in each cage. The following formula was used:

$$\text{Daily food consumption (g/rat/day)} = \frac{\text{Food given to cage - food left by cage}}{\text{Number of animal days *}}$$

The results using this formula (presented in Appendix 3) were subject to rounding to the nearest whole number.

To provide a more accurate measure of the food consumed by each animal in a group, the following formula was used for group mean calculation over each period:

$$\text{Daily group mean food consumption (g/rat/day)} = \frac{\text{Total food given to group - total food left by group}}{\text{Number of animal days * for the group}}$$

It is therefore inappropriate to attempt to calculate the stated group means (presented in Table 2) from the individual values in the appendix.

* The term 'animal day' counts one animal day for each animal alive for a whole day. Thus in a 3-animal cage with total survival over a 4 day period, there are 12 (3 animals x 4 days) animal days of consumption. It is assumed that on the day of death and animal does not eat.

TERMINAL STUDIES

Necropsy

All adult female animals were killed by carbon dioxide asphyxiation on Day 20 of gestation. Immediately prior to necropsy the animals were weighed.

A macroscopic examination of all rats was performed according to the following detailed necropsy procedure.

The abdomen of each animal was dissected and examined for congenital abnormalities and macroscopic pathological changes in the maternal organs. Abnormal tissues were preserved at the discretion of the pathologist. The ovaries and uteri were examined immediately to determine:

Number of corpora lutea
Number of implantation sites
Number of resorption sites (early, late and total)
Number and distribution of embryofoetal deaths
Individual foetal weight from which the litter weight was calculated
Individual placentae weights
Foetal abnormalities

Embryo-foetal deaths were classified as:

Early: only placenta visible at termination
Late: both placenta and embryonic remnants visible at termination

All foetuses were subjected to an external examination and the sex recorded. Each foetus was individually identified according to location within the litter. Following external examination all foetuses were killed by chilling on a cold plate (a recognised method of sacrifice), then discarded.

TREATMENT OF DATA

Where appropriate, group mean values are shown as means and standard deviations.

Food consumption data is presented as individual cage values and group mean values over the periods recorded. Cumulative data over appropriate periods is reported.

Bodyweight is presented as individual and mean values. Bodyweight change values are presented over appropriate periods.

Litter parameters

The standard unit of assessment was the litter, therefore the group mean values are calculated from individual litter values.

$$\text{Pre - implantation loss} = \frac{\text{Number of corpora lutea - Number of implanations} \times 100}{\text{Number of corpora lutea}}$$

$$\text{Post - implantation loss} = \frac{\text{Number of implantations - Number of viable foetuses} \times 100}{\text{Number of implantations}}$$

Group mean litter size

Group mean litter size and SD were calculated from individual litter values.

Group mean foetal bodyweight

Group mean foetal offspring bodyweight and SD was calculated separately for male, female and overall:

$$\text{Foetal weights} = \frac{\text{Total number of individual litter mean foetal weights}}{\text{Number of litters}}$$

Group mean placental weights

Group mean values and SD were calculated from:

$$\text{Placental weights} = \frac{\text{Total of individual litter placental weights}}{\text{Number of litters}}$$

Sex ratio

For each group the ratio of male to female offspring was calculated for all offspring. The ratio was expressed as the following formulae:

$$\frac{\text{Number of males in litter}}{\text{Total number of offspring in litter}} \times 100$$

STATISTICAL ANALYSIS

The small sample size precluded meaningful statistical evaluation. Inter-group differences were assessed by reference to control data.

ARCHIVING

All raw data generated by the Sponsor has been retained in the Sponsor's archives.

All specimens, raw data and study-related documents generated during the course of the study at Huntingdon Life Sciences, together with a copy of the final report were lodged in Huntingdon Life Sciences, Archives.

Such specimens and records will be retained for a minimum period of ten years, from the date of issue of the final report. At the end of the ten-year retention period the Sponsor will be contacted and advice sought on the future requirements. Under no circumstances will any item be discarded without the Sponsor's knowledge.

DEVIATION FROM PROTOCOL AND STANDARD OPERATIONS PROCEDURES

Food consumption was measured from Days 2-3 and 4-7, instead of 2-4 and 5-7 to correspond with the bodyweights.

The sex ratio was reported in addition to protocol-driven data handling.

The fetuses were killed by cold plate euthenasia, not be an injection of sodium pentobarbital as stated in the protocol.

There were no significant deviations from Standard Operating Procedures.

These deviations are considered to have no impact on the integrity of the study.

RESULTS

CHAMBER ATMOSPHERE CONDITIONS

Chamber analysed concentration of 2-methyl-2-butene

The data are presented in **ADMINISTRATION OF 2-METHYL-2-BUTENE BY INHALATION TO RATS** appended to this report.

The data are summarised below:

Group	Study mean concentration ppm	
	Target	Analysed
2 (Low dose	2000	1971
3 (Inter dose)	4000	4027
4 (High dose)	7000	7109

The analysed mean concentrations were in agreement with the target concentrations. On one occasion (Day 6) the low dose level was markedly lower than target, but this, in isolation, was considered to have no impact on the assessment of study results.

CLINICAL OBSERVATIONS

Mortality

There were no unscheduled deaths.

Clinical signs (Appendix 1)

There were no adverse signs seen during the course of the study.

Bodyweight (Figure 1, Table 1, Appendix 2)

Group mean bodyweight gain in all treated groups was transiently lower than the concurrent control group during the first 4 days of exposure (Days 12 to 16 of gestation). However since there was no dosage relationship and bodyweight gain from Day 16 of pregnancy was essentially comparable with the control, this change is of uncertain toxicological significance.

Food consumption (Table 2, Appendix 3)

Following the start of treatment group mean food consumption was lower than the controls in all treated groups. This effect tended to correlate with the effect on bodyweight and is of uncertain relationship to treatment.

TERMINAL STUDIES

Macroscopic examination - adult females

There were no macroscopic changes detected at post mortem examination of any female on Day 20 of pregnancy.

Litter values and external foetal examination (Tables 3 and 4, Appendices 4 and 5)

In Groups 1 to 4 respectively all females had live young at Day 20 for assessment, providing 79, 79, 77 and 82 foetuses.

Implantations and litter data

There were no treatment-related effects on the number of implantations or subsequent litter size. Implantation losses were low and there was no evidence of the selective loss of either sex, as evidenced by a similar sex ratio in all groups.

Foetal, placental and litter weights

Foetal, litter and placental weights were unaffected by treatment.

Macroscopic foetal examination

There were no gross macroscopic changes detected at external examination of foetuses from females killed on Day 20 of pregnancy.

DISCUSSION AND CONCLUSION

Within the context of this study, 2-methyl-2-butene was very well tolerated when administered to pregnant rats on Days 12 to 19 of gestation by inhalation by whole body exposure at concentrations of 2000, 4000 and 7000 ppm.

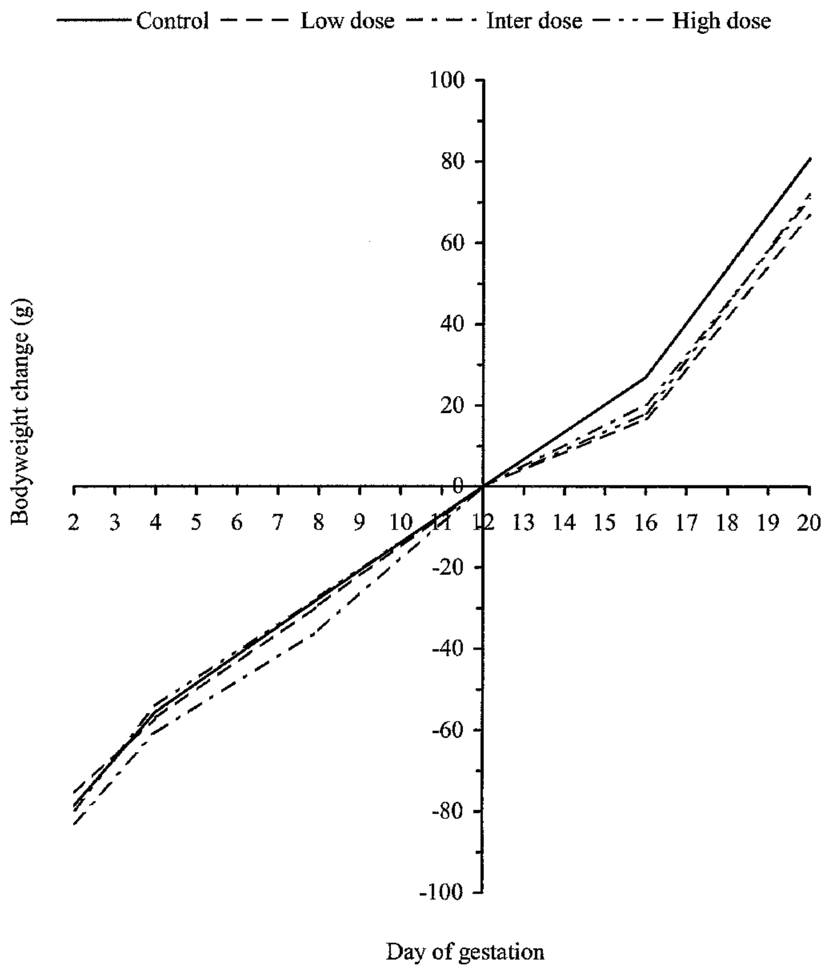
Bodyweight gain and food consumption of females was slightly lower in treated groups of rats following the start of exposures but there was no dosage relationship. There were no effects upon the growth and survival of the foetuses.

It was thus concluded that animals would well tolerate exposures up to 7000 ppm in the subsequent OECD 422 study in the rat.

FIGURE 1

Bodyweight change - group mean values

Group:	1	2	3	4
Compound:	Control	----2-methyl-2-butene----		
Dosage (ppm):	0	2000	4000	7000



Treatment commenced Day 12
Change values are relative to start of treatment

TABLE 1

Bodyweight and bodyweight change - group mean values - adult females

Group:	1	2	3	4
Compound:	Control	----2-methyl-2-butene----		
Dosage (ppm):	0	2000	4000	7000

Group		Bodyweight (g) on Day of gestation					
		2	4	8	12	16	20
1	Mean	228	251	279	306	333	387
	SD	12	16	17	20	22	34
	n	6	6	6	6	6	6
2	Mean	221	240	268	297	314	364
	SD	10	15	18	25	32	45
	n	6	6	6	6	6	6
3	Mean	230	252	277	313	333	383
	SD	9	13	10	10	12	19
	n	6	6	6	6	6	6
4	Mean	223	249	275	303	321	375
	SD	12	14	14	20	17	19
	n	6	6	6	6	6	6

SD Standard deviation

Group		Bodyweight change (g) relative to start of treatment on Day of gestation					
		2	4	8	12	16	20
1	Mean	-79	-56	-28	0	27	81
2	Mean	-76	-57	-29	0	17	67
3	Mean	-83	-61	-36	0	20	71
4	Mean	-80	-54	-27	0	18	72

Treatment commenced Day 12

TABLE 2

Food consumption - group mean values - adult females

Group:	1	2	3	4
Compound:	Control	----2-methyl-2-butene----		
Dosage (ppm):	0	2000	4000	7000

Group		Food consumed during days of gestation (g/rat/day)				
		2-3	4-7	8-11	12-15	16-19
1	Mean	30	29	30	34	32
	SD	3	1	2	5	5
	N	2	2	2	2	2
2	Mean	28	28	31	27	29
	SD	2	1	1	1	0
	N	2	2	2	2	2
3	Mean	29	28	30	27	29
	SD	0	2	2	1	3
	N	2	2	2	2	2
4	Mean	28	27	31	26	29
	SD	2	2	1	2	0
	N	2	2	2	2	2

SD Standard deviation
Treatment commenced Day 12

TABLE 3

Implantations and litter size - group mean values

Group:	1	2	3	4
Compound:	Control	----2-methyl-2-butene----		
Dosage (ppm):	0	2000	4000	7000

Group		Corpora Lutea	Implantations	Resorptions			Live young			Sex ratio (% M)	Implantation loss (%)	
				Early	Late	Total	Male	Female	Total		Pre-	Post-
1	Mean	14.0	13.3	0.2	0.0	0.2	7.0	6.2	13.2	53.4	6.4	1.0
	SD	1.3	2.6				2.1	2.4	2.4			
	n	6	6	6	6	6	6	6	6	6	6	6
2	Mean	15.5	13.8	0.7	0.0	0.7	7.3	5.8	13.2	55.0	11.7	4.7
	SD	1.0	1.9				2.3	1.6	1.8			
	n	6	6	6	6	6	6	6	6	6	6	6
3	Mean	15.0	14.3	1.5	0.0	1.5	6.3	6.5	12.8	49.0	4.3	10.3
	SD	1.7	1.5				3.4	3.4	1.9			
	n	6	6	6	6	6	6	6	6	6	6	6
4	Mean	14.3	13.8	0.2	0.0	0.2	7.8	5.8	13.7	57.3	4.4	1.3
	SD	1.5	1.5				1.5	1.3	1.6			
	n	6	6	6	6	6	6	6	6	6	6	6

SD Standard deviation
% M % Males/litter

TABLE 4

Fetal,plaental and litter weights - group mean values

Group:	1	2	3	4
Compound:	Control	----2-methyl-2-butene----		
Dosage (ppm):	0	2000	4000	7000

Group		Placental weight (g)	Litter weight (g)	Foetal weights (g)		
				Males	Females	Overall
1	Mean	0.56	53.87	4.16	4.00	4.09
	SD	0.06	10.03	0.09	0.10	0.07
	n	6	6	6	6	6
2	Mean	0.52	50.52	3.93	3.74	3.84
	SD	0.03	7.03	0.22	0.14	0.16
	n	6	6	6	6	6
3	Mean	0.55	49.79	3.99	3.80	3.91
	SD	0.03	6.51	0.33	0.34	0.34
	n	6	6	6	6	6
4	Mean	0.52	53.10	3.96	3.78	3.89
	SD	0.06	7.07	0.31	0.28	0.31
	n	6	6	6	6	6

SD Standard deviation

APPENDIX 1

Clinical signs and necropsy - individual findings - adult females

Group:	1	2	3	4
Compound:	Control	----2-methyl-2-butene----		
Dosage (ppm):	0	2000	4000	7000

Group	Animal number	Clinical signs observed (Day of gestation)	Necropsy findings
1	1	-	NAD
	2	-	NAD
	3	-	NAD
	4	-	NAD
	5	-	NAD
	6	-	NAD
2	7	-	NAD
	8	-	NAD
	9	-	NAD
	10	Brown staining head, forelimbs and upper dorsal thorax (D20)	NAD
	11	-	NAD
	12	-	NAD
3	13	Slight brown staining to head and forelimbs (D20)	NAD
	14	-	NAD
	15	-	NAD
	16	-	NAD
	17	-	NAD
	18	-	NAD
4	19	-	NAD
	20	-	NAD
	21	-	NAD
	22	-	NAD
	23	-	NAD
	24	-	NAD

-   No clinical signs evident
NAD   No abnormalities detected

APPENDIX 2

Bodyweights - individual values - adult females

Group:	1	2	3	4
Compound:	Control	----2-methyl-2-butene----		
Dosage (ppm):	0	2000	4000	7000

Group	Animal number	Bodyweight (g) on day of gestation					
		2	4	8	12	16	20
1	1	222	248	273	293	323	386
	2	237	259	288	316	344	394
	3	248	278	309	341	373	450
	4	216	236	262	292	316	371
	5	222	247	275	305	331	361
	6	221	237	265	291	313	361
2	7	205	220	241	264	272	309
	8	223	243	272	302	327	372
	9	224	244	273	300	321	386
	10	229	246	281	316	341	407
	11	233	260	287	327	344	402
	12	214	226	251	272	277	306
3	13	236	264	295	331	341	389
	14	219	240	267	312	332	397
	15	237	261	280	303	337	356
	16	225	237	274	306	310	364
	17	241	266	273	315	338	396
	18	221	244	274	309	340	398
4	19	226	255	291	328	344	401
	20	224	256	283	313	329	379
	21	216	246	267	300	318	376
	22	214	242	265	286	309	367
	23	244	266	289	314	328	382
	24	214	227	257	275	297	343

Treatment commenced Day 12

APPENDIX 3

Food consumption - individual values - adult females

Group:	1	2	3	4
Compound:	Control	----2-methyl-2-butene----		
Dosage (ppm):	0	2000	4000	7000

Group	Cage number	Food consumed during days of gestation (g/rat/day)				
		2-3	4-7	8-11	12-15	16-19
1	1	33	30	31	37	36
	2	28	28	29	30	29
2	3	26	27	32	26	29
	4	29	28	30	28	29
3	5	29	27	28	26	28
	6	28	29	31	27	33
4	7	30	29	30	27	29
	8	27	26	32	25	29

Treatment commenced Day 12

APPENDIX 4

Implantations and litter siz e - individual values

Group:	1	2	3	4
Compound:	Control	----2-methyl-2-butene----		
Dosage (ppm):	0	2000	4000	7000

Group	Animal number	Corpora lutea	Implants	Resorptions			Live young			Sex ratio (% M)	Implantation loss (%)	
				Early	Late	Total	Male	Female	Total		Pre-	Post-
1	1	14	15	0	0	0	7	8	15	46.7	0.0	0.0
	2	13	9	0	0	0	4	5	9	44.4	30.8	0.0
	3	16	16	1	0	1	5	10	15	33.3	0.0	6.3
	4	13	12	0	0	0	8	4	12	66.7	7.7	0.0
	5	15	15	0	0	0	9	6	15	60.0	0.0	0.0
	6	13	13	0	0	0	9	4	13	69.2	0.0	0.0
2	7	16	12	0	0	0	5	7	12	41.7	25.0	0.0
	8	14	11	1	0	1	5	5	10	50.0	21.4	9.1
	9	15	15	1	0	1	7	7	14	50.0	0.0	6.7
	10	15	16	2	0	2	7	7	14	50.0	0.0	12.5
	11	16	15	0	0	0	9	6	15	60.0	6.3	0.0
	12	17	14	0	0	0	11	3	14	78.6	17.6	0.0

% M % Males/litter

APPENDIX 4

(Implantations and litter siz e - continued)

Group:	1	2	3	4
Compound:	Control	----2-methyl-2-butene----		
Dosage (ppm):	0	2000	4000	7000

Group	Animal number	Corpora lutea	Implants	Resorptions			Live young			Sex ratio (% M)	Implantation loss (%)	
				Early	Late	Total	Male	Female	Total		Pre-	Post-
3	13	15	15	2	0	2	9	4	13	69.2	0.0	13.3
	14	18	17	3	0	3	2	12	14	14.3	5.6	17.6
	15	15	14	0	0	0	5	9	14	35.7	6.7	0.0
	16	14	13	4	0	4	3	6	9	33.3	7.1	30.8
	17	15	14	0	0	0	9	5	14	64.3	6.7	0.0
	18	13	13	0	0	0	10	3	13	76.9	0.0	0.0
4	19	14	13	1	0	1	7	5	12	58.3	7.1	7.7
	20	15	16	0	0	0	9	7	16	56.3	0.0	0.0
	21	14	14	0	0	0	10	4	14	71.4	0.0	0.0
	22	13	13	0	0	0	6	7	13	46.2	0.0	0.0
	23	17	15	0	0	0	8	7	15	53.3	11.8	0.0
	24	13	12	0	0	0	7	5	12	58.3	7.7	0.0

% M % Males/litter

APPENDIX 5

Foctal,placental and litter weights - individual values

Group:	1	2	3	4
Compound:	Control	----2-methyl-2-butene----		
Dosage (ppm):	0	2000	4000	7000

Group	Animal number	Placental weights (g)		Litter weight (g)	Foetal weights (g)					
					Males		Females		Total	
		Mean	SD		Mean	SD	Mean	SD	Mean	SD
1	1	0.45	0.04	60.39	4.12	0.15	3.94	0.15	4.03	0.17
	2	0.57	0.05	36.54	4.11	0.11	4.02	0.09	4.06	0.10
	3	0.59	0.09	61.91	4.21	0.16	4.09	0.17	4.13	0.17
	4	0.61	0.06	49.93	4.17	0.15	4.14	0.28	4.16	0.19
	5	0.53	0.08	62.57	4.32	0.19	3.95	0.15	4.17	0.25
	6	0.58	0.04	51.89	4.04	0.15	3.87	0.12	3.99	0.16
2	7	0.48	0.04	43.77	3.66	0.20	3.64	0.14	3.65	0.16
	8	0.55	0.06	39.87	4.09	0.12	3.88	0.09	3.99	0.15
	9	0.54	0.04	55.68	4.05	0.11	3.91	0.20	3.98	0.17
	10	0.56	0.06	55.90	4.19	0.15	3.79	0.18	3.99	0.26
	11	0.49	0.05	56.10	3.85	0.21	3.58	0.20	3.74	0.24
	12	0.52	0.05	51.82	3.72	0.18	3.65	0.10	3.70	0.17

APPENDIX 5

Foetal,placental and litter weights - continued)

Group:	1	2	3	4
Compound:	Control	----2-methyl-2-butene----		
Dosage (ppm):	0	2000	4000	7000

Group	Animal number	Placental weights (g)		Litter weight (g)	Fetal weights (g)					
					Males		Females		Total	
		Mean	SD		Mean	SD	Mean	SD	Mean	SD
3	13	0.51	0.06	46.57	3.66	0.22	3.41	0.07	3.58	0.22
	14	0.54	0.05	53.92	3.91	0.22	3.84	0.11	3.85	0.12
	15	0.53	0.07	48.64	3.62	0.22	3.40	0.36	3.47	0.33
	16	0.57	0.05	38.82	4.41	0.42	4.26	0.04	4.31	0.23
	17	0.56	0.06	55.59	4.01	0.19	3.90	0.15	3.97	0.18
	18	0.57	0.10	55.17	4.32	0.18	3.98	0.34	4.24	0.26
4	19	0.49	0.08	51.39	4.33	0.17	4.21	0.27	4.28	0.21
	20	0.60	0.16	59.56	3.78	0.27	3.64	0.14	3.72	0.23
	21	0.50	0.06	58.62	4.30	0.19	3.92	0.18	4.19	0.25
	22	0.48	0.08	45.58	3.57	0.21	3.45	0.23	3.51	0.22
	23	0.58	0.06	59.27	4.02	0.12	3.88	0.19	3.95	0.17
	24	0.46	0.04	44.16	3.76	0.10	3.56	0.16	3.68	0.16

APPENDIX 6

Certificate of analysis for 2-Methyl-2-Butene

CERTIFICATE OF ANALYSIS

DEPARTMENT OF PRODUCT CHEMISTRY (HUNTINGDON)

HUNTINGDON LIFE SCIENCES

Test substance:	2-Methyl-2-butene
Batch number:	A0153320
Analysis dates:	Initial analysis: 2 August 2001 Final analysis: 12 December 2002
Data obtained as part of study:	CSS/007

Purity determined by GC analysis.

Purity:	Initial analysis:	98.0 %w/w
	Final analysis:	97.8 %w/w

Test substance stable for the duration of work performed at Huntingdon Life Sciences.

J. M. Betteley
John Betteley,
Study Director,
Huntingdon Life Sciences Ltd.

11 June 2003
Date

ADMINISTRATION OF 2-METHYL-2-BUTENE

BY INHALATION TO RATS

Author
Simon Moore

CONTENTS

TEST SUBSTANCE AND ADMINISTRATION

TEST SUBSTANCE

The test substance, 2-methyl-2-butene, is a volatile liquid with boiling point of between 35 and 38°C.

A consignment, comprising of five steel drums, each with a stated net content of 35 kg (Lot number A0153320), was received from Acros Organics on 16 May 2001. The test substance was stored securely in the original containers at room temperature until it was transferred to the atmosphere generation system.

Information provided indicated that the test substance was stable for the intended duration of use on the study. The stated purity was 98.2%. This was confirmed by reanalysis following completion of the study (see Appendix 6).

ADMINISTRATION

The test material was administered to the rats by inhalation in whole-body exposure chambers as described below:

The chamber atmospheres were produced by metering the liquid test substance into glass vapour generators through which dried air was passed at a group dependent flow rate ranging from 30 to 150 l/minute. The atmosphere produced by the generation system was (except for Group 4) further diluted with air to give a total flowrate of 150 l/minute and to give the final chamber concentrations of test aerosol.

The in-line airflow to the vapour generation apparatus was verified using a dry type gas meter during the preliminary phase of the study. During the study, the airflow to the atmosphere generation system was monitored throughout each of the exposures using calibrated in-line tapered tube gas flowmeters.

The settings of the test substance metering system required to obtain the target chamber concentrations were determined during preliminary generation trials without animals present and based on the gas chromatographic (GC) analysis of chamber atmosphere samples. Minor adjustments were made to the test material delivery rates in order to maintain chamber concentrations close to target.

Animals assigned to Group 1 (Air control) received an exposure to compressed air only, from the same source as used for the generation of the test atmospheres.

The duration of administration was a single 6-hour exposure, daily, for 8 days.

The usage of 2-methyl-2-butene was determined, for each day of treatment, for each test group.

TEST ATMOSPHERE GENERATION

The vapour generation system for each of the test groups was supplied from individual reservoirs of liquid 2-methyl-2-butene maintained at pressure. The top of each reservoir[1] was fitted with a central, "O" - ring sealed filler cap, a system to allow pressurisation and release of the helium head pressure and a safety pressure release valve set to operate at above the study operating pressure. The reservoirs were mounted on electronic load cells[2] and the weight of each reservoir and contents could be displayed continuously. Each load cell was set to read zero weight with the empty reservoir in place before the first occasion of filling with the test substance and the minimum permissible start weight for the study exposures was calculated. Except during the filling procedure, 2-methyl-2-butene in the reservoirs was maintained under a helium pressure of 10 psi for groups 2, 3 and 4 (Low, Intermediate and High dose).

The pressurised reservoirs supplied the test substance to all vapour generators comprising of a glass frit contained in a glass vessel. For each test group, the test substance delivery rate was controlled using a metering valve (Nupro S Series Needle Valve[3]). The liquid delivery line to each metering valve was fitted with a toggle valve to allow isolation of the test substance supply. The liquid delivery line was also fitted with a particulate filter (stainless steel, 90 μm pore size[3]) upstream of each metering valve, to protect the valve from any entrained particulate. Fluid passing through each metering valve was delivered onto the glass frit surface of the vaporiser, at ambient temperature. The air supply (30 to 150 l/minute) for the generator was first passed through a separate copper coil, at ambient temperature. The test substance vapour and air mixture was passed through fibre reinforced PVC tubing (10mm internal diameter). Care was taken to ensure that the lower explosive limit (14,000 ppm; established in report number 22101 by Chilworth Technology, Southampton, UK.) was never exceeded in the generation system.

For all groups exposed to 2-methyl-2-butene, the vapour/air mixture produced in the vapour generators was passed into the base of the secondary dilution vessel. A further supply of clean and dry air was supplied to Groups 2 and 3 to ensure a total chamber airflow of approximately 150 l/minute. The air supply for Group 4 was provided solely by the vapour generation system.

Diluent air flow was measured using a tapered tube flow meter situated at the front of a purpose-built stainless steel trolley on which the secondary dilution vessel was mounted. Generation air was measured on a similar flowmeter mounted on the vapour generation trolley.

The test atmosphere was then passed through flexible ducting to a tangential inlet mounted at the apex of the appropriate exposure chamber.

A schematic of the vapour generation system is presented in Figure A.

The control group was exposed using a similar system to that used for the test groups, but received compressed air only at a rate of approximately 150 l/minute.

The air supplied to the vapour generators and secondary dilution vessels was filtered to remove any residual particulate and was dried (dew point ~2°C).

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1 Newson Gale Ltd, 51 Norsey Road, Billericay, Essex, CM11 1BG, England

2 Huntleigh Industrial Controls Ltd, Load Cell Division, Portman Moor Industrial Estate, East Moors, Cardiff, South Glamorgan, CF22 2HB

3 Nupro Co, Willoughby, Ohio 44094, USA

EXPOSURE CHAMBERS

The exposure chambers were of stainless steel and glass construction and consisted of a cuboidal body fitted with a pyramidal base and top. The internal volume of each chamber was approximately 0.75 m^3. At the apex of the upper pyramidal figure was the tangentially mounted air duct. Immediately below this was a perforated canister, which ensured equal distribution of the test atmosphere within the chamber.

Access to the chamber was through the front of the box section *via* a hinged door with a glass panel and stainless steel frame. The door was sealed using moulded rubber sealing strip.

Exposure cages constructed of stainless steel mesh were suspended on a framework arranged on 4 levels. Each level is able to hold four cages, with each cage capable of housing 4 rats individually. This gave a potential animal exposure capacity of 64 rats. In this investigation, 6 animal compartments were used on level 2 and no cages were present on levels 1, 3 or 4.

Projecting through the rear wall of each chamber was one 0.25 inch diameter stainless steel tube protruding between levels 2 and 3 of the chamber. This was used for collection of chamber atmosphere samples. Spatial distribution studies were conducted during preliminary trials.

The pyramidal base of each chamber was fitted with a 2-inch drain. The drain connected with a common drainage system *via* a ball valve.

A square tubular exhaust plenum, 3 inches in diameter and perforated along the ventral surface, was situated in the pyramidal base. This connected to the main extract system.

A wet and dry alcohol bulb thermohygrometer was suspended in the chamber. This was visible through the glass-panelled door and was used to monitor chamber temperature and relative humidity.

A Magnehelic pressure gauge (0 - 25 mm water gauge) was connected with each chamber by a nylon tube. This was mounted on the secondary dilution vessel trolley and was used to monitor the atmosphere pressure inside the chamber, relative to the exposure room. The internal pressure within each chamber was maintained in the range -2 to -4 mm water below ambient pressure when operational.

Extract flow was adjusted using gate valves mounted in the extract ducting between the chamber and filters.

Extraction of the chambers was accomplished by means of a single fan mounted on the outside wall of the building withdrawing air through a manifold to which all chambers were connected. The chamber air extract was vented to atmosphere *via* an exhaust stack.

A schematic of the exposure chambers is presented in Figure B.

PROCEDURE

A separate exposure chamber was used for each group. The Control animals were exposed using an identical exposure chamber to that used for the test groups.

Prior to the start of each exposure, the mass of test substance in each of the pressure vessels was checked to ensure there was sufficient material for the scheduled duration of generation.

The rats were transferred from the holding cages and placed into the individual compartments of the exposure cages. The animals were located on level 2 of the chamber with the position of the animals within the chamber unaltered throughout the duration of the study.

The diluent and generator airflows were turned on and the exposure chamber doors were checked to ensure they were secured. The chamber pressure, relative to the exposure room was checked using each of the associated Magnehelic gauges to ensure that operation of the chamber took place at a slightly negative pressure.

The test substance supply toggle valves between the pressure vessel and the metering valves were opened; the exposure start time noted and simultaneously the chamber environmental monitoring system was activated (see below). At intervals of 30 minutes, any reactions by the rats to exposure were recorded together with checks of generation and chamber operational parameters.

The wet and dry bulb temperatures of a thermohygrometer placed in each chamber were also recorded at approximately 30-minute intervals throughout each exposure. Relative humidity was found using a look-up table. The volume flow of air to the exposure chambers was measured using calibrated flow meters and also checked approximately every 30 minutes.

Results of the determination of 2-methyl-2-butene in the chamber atmosphere were automatically recorded for each chamber at approximately half hourly intervals throughout each exposure.

At the end of six hours generation, the isolating toggle valves in the test substance supply lines were turned off and the weight of each pressure vessel and its residual contents was recorded. The vapour in the test chambers was allowed to clear for at least 15 minutes before the animals were removed.

At the end of this time, the rats were unloaded from the chambers and returned to their respective holding cages.

The chambers were washed with hot water.

A summary of the operating conditions used is presented in Table A.

AEROSOL ANALYSIS

A gas chromatograph was used to measure the concentrations of 2-methyl-2-butene in the test atmospheres within the four-inhalation chambers. Operating details of the Gas Chromatography system, its standardisation and validation are given in Appendix A.

The Gas Chromatograph was located adjacent to the exposure chambers.

The instrument was connected to each selected sampling port by programmed switching of valves under the control of the CEMS-2 program. Gas sampling lines were 0.6 cm diameter stainless steel tubing. A further set of automated valves admitted standards in gas sampling bags for calibration of the Gas Chromatograph and for daily checking of the standard response. To minimise the opportunity for carry over of the test substance within the sample lines, the conduit in which the sample stream passed to the Gas Chromatograph was purged for 60 seconds between analyses.

Before the start of each exposure, the operating conditions for the Gas Chromatograph were identified and the instrument response checked using prepared standard gas mixtures. An automatic warning message was generated by any deviation from the accepted response range for the standards.

Linear regression analysis of the Gas Chromatograph response to standards was incorporated into the system program to enable concentrations to be calculated from the signals provided by the chromatograph. The accumulated calibration data were reviewed at intervals during the study and, if necessary, the regression data incorporated into the program were revised. Details of such reviews are retained with the raw data.

CHAMBER MONITORING SYSTEM

A PC running the Chamber Environmental Monitoring System (CEMS-2) software was used to monitor and record the system performance during each exposure. The data collection sequence and display were controlled by a personal computer (PC) and all information collected was displayed on a monitor. Simultaneously, the data was stored electronically. This program was composed of three basic stages of operation: an initial setting up (pre-exposure) phase, an exposure monitoring phase and the post exposure data collation and presentation phase. The program is driven by a study data-protocol containing study specific design and detail. The CEMS-2 system holds a certificate of validation in compliance with GLP. All information collected was printed as a hard copy.

Setting-up phase

In the initial phase, prompted by the program screen display, study identification, dates, times and other relevant study details together with barometric pressure, airflow transducer calibration, gas chromatograph calibration and actual chamber airflow data sets were entered and stored.

Exposure monitoring phase

This phase was started coincident with the commencement of generation. Each chamber's environment was monitored during a 30-minute cycle, during the analysed concentration was recorded. The data were displayed on screen, printed and stored on disc. This cycle of monitoring took place in the following sequence: High dose, Intermediate dose, Low dose and Air control and was repeated throughout the six-hour exposure period. A total of twelve cycles were recorded.

Post exposure phase

At the end of 6 hours, the data collected during exposure were collated into separate groups. The mean values, together with standard deviation were calculated for each parameter recorded. This data were printed and stored on disc. The first set of data was excluded from calculation of the mean because chamber concentrations did not stabilise until approximately 15 - 20 minutes from the start of exposure (equilibration time, t_{99} was 23 minutes).

TARGET CONCENTRATIONS

The target concentrations of 2-methyl-2-butene were:

Group	Designation	Concentration (ppm)
2	Low dose	2000
3	Inter dose	4000
4	High dose	7000

The target concentrations were selected in consultation with the Sponsor, following the review of available data.

EXPOSURE CHAMBER CONDITIONS

Chamber analysed concentration of 2-methyl-2-butene

Chamber atmosphere was sampled in sequence from each of the four exposure chambers (Chambers 4 - 1 sampled sequentially) and from one point within each chamber. Air from each chamber was continually drawn through a transfer line, which was therefore equilibrated with the mean concentration from each chamber. When not being sampled, these transfer lines were pumped to waste (Figure B).

Every seven minutes, air from the transfer lines was switched to the injection loop of the gas chromatograph for automated analysis and data logging.

The analytical methodology is presented in Appendix A.

Chamber spatial distribution

Chamber spatial distribution was conducted during preliminary trials. A minute difference in concentration with varying sample point was observed, which was well within the 10% tolerance limits normally allowed for this type of system. The sample points are presented in Figure B.

Nominal concentration of chamber atmospheres

Each chamber nominal concentration was calculated from the mass of liquid used over the six-hour exposure period and the exposure mean airflow. The ideal gas equation was used with the molecular weight of the liquid and the measured chamber conditions to compute the volume of vapour produced from the mass of liquid used. The calculation is detailed in Table C.

The chamber nominal concentrations were calculated from the amount of liquid used over the six-hour exposure period, the mass of the liquid and the exposure mean airflow. The formulae used were as follows:

$$\text{Concentration} = \frac{V}{Va + V} \times 1{,}000{,}000 \text{ ppm} \qquad (1)$$

$$V = \frac{W \times R \times T}{M} \times \frac{760}{Atm} \qquad (2)$$

where	V	=	gaseous volume of 2-methyl-2-butene (L)
	W	=	mass of 2-methyl-2-butene (g)
	M	=	molecular weight of 2-methyl-2-butene (70.14 g/mole)
	R	=	Gas constant (0.08205 L atm mol^{-1} K^{-1})
	T	=	temperature (K)
	Atm	=	atmospheric pressure (mmHg)
	V_a	=	volume of air (L)

Airflow, temperature and relative humidity

These parameters were recorded manually, as described above under the Test Atmosphere generation and Procedure sections.

RESULTS

VAPOUR CONCENTRATION

Analysed concentration of 2-methyl-2-butene

The data are presented as follows:

Daily mean values	Table B
Individual values	Appendix B

The study mean concentration (the mean of daily mean values) for each group exposed to 2-methyl-2-butene are presented below:

Group	Chamber concentration (ppm)	
	Target	Analysed
2 (Low dose)	2000	1971
3 (Inter dose)	4000	4027
4 (High dose)	7000	7109

Analysed mean concentrations were in good agreement with target concentrations. The coefficients of variation of the daily means were 16.1, 6.2 and 5.3 % for Groups 2, 3 and 4 respectively. On one occasion (Day 6) the low dose level was markedly lower than target, but this, in isolation, was considered to have no impact on the assessment of study results.

Nominal concentration of 2-methyl-2-butene

The data are presented in Table C and are summarised below:

Group	Nominal concentration (ppm)	A/N ratio (%)
2 (Low dose)	1885	105
3 (Inter dose)	4384	92
4 (High dose)	7055	101

$$A/N = \left(\frac{\text{Analysed concentration}}{\text{Nominal concentration}}\right) \times 100$$

For each Group, the nominal concentration for each exposure was calculated from the following parameters:

The mass of 2-methyl-2-butene delivered into each vapour generator;
The mean chamber temperature;
The barometric (atmospheric) pressure;
The molecular weight of 2-methyl-2-butene;
The gas constant;
The chamber airflow;
The exposure duration.

The equations used for the calculation of the nominal concentration are detailed in Table C.

The mean ratios of analysed to nominal concentration (A/N), expressed as a percentage for the study, were between 92 and 105%. The A/N ratios for all dose groups were within the acceptable tolerances for the dynamic vapour generation system used in this study. Differences from the ideal A/N of 100% may be due to inaccuracies in the measurements of weight, airflow, temperature and analysed concentrations.

CHAMBER TEMPERATURE AND RELATIVE HUMIDITY

The daily mean chamber temperatures and relative humidities are presented in Table D.

The chamber temperatures were similar for all groups for most days of the study.

For Group 2 (Low dose), the recorded RH was at the low end of the target range of 40 - 60%. However, for Groups 1, 3 and 4 (Air control, Intermediate and High dose), the relative humidity (RH) measurements recorded during the study were about 10% lower. The low values of RH probably arise from generation and dilution of the chamber atmospheres with air that was supplied from a compressor system incorporating a refrigerant drier. This deviation from the target conditions had no discernible effect upon the animals and is not considered to have affected the outcome of the study.

DISCUSSION

Control of the 2-methyl-2-butene vapour delivery to the exposure chambers was very good, as reflected in the study mean concentrations, which were within 2% of the target values for all groups.

The coefficients of variation for the daily mean concentrations were 16.1, 6.2 and 5.3% for Groups 2, 3 and 4 respectively. The greater variation in daily mean concentrations observed in Group 2 (Low dose) exposures was considered to reflect the difficulty of repeatably delivering a very low flow rate (ca. 1.3 ml/min) of volatile material to the vaporiser.

All individual samples in all exposures, collected from the Group 1 (Air control) chamber showed traces of 2-methyl-2-butene. This was considered to be a "carryover" effect in the common sampling line from the preceding sample rather than the presence of 2-methyl-2-butene within the exposure chamber. The mean amount present in terms of the peak area response was (on average) equivalent to 0.2% of the area response of the preceding Group 2 (Low dose) sample (target concentration 2000 ppm).

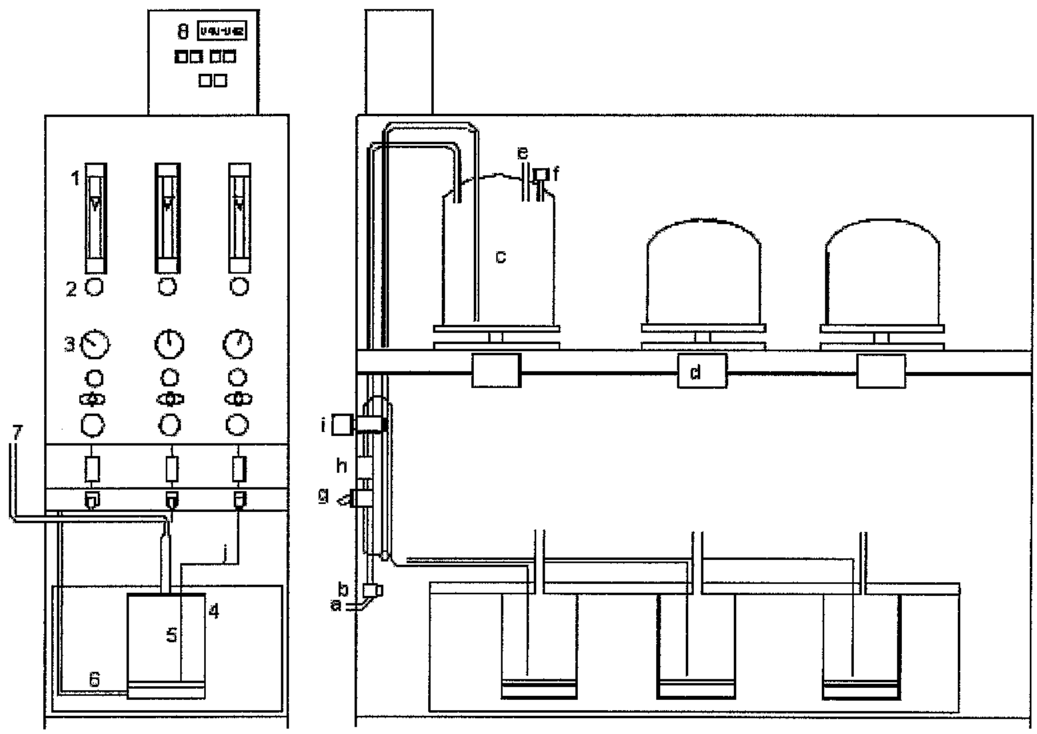
Good agreement was also observed between the analysed and the nominal chamber concentration values for Groups 2 and 4 (Low and high doses respectively) where study mean analysed/nominal (A/N) ratios of 105 and 101% were observed. For Group 3 (Intermediate dose) the A/N ratio was 92% reflecting a possible error in airflow measurement or a leak of air into the chamber as the coefficient of variations are small, suggesting that the generation was well controlled.

CALCULATIONS

In order to minimise the cumulative errors which result from repeated rounding of numbers, much of the data in this report has been calculated continuously using unrounded numbers and only rounded for printing. Consequently, any further calculations using these rounded numbers may include rounding errors in the last significant figure, possibly leading to small apparent discrepancies with other data in the report.

FIGURE A

Schematic of a vapour generation system

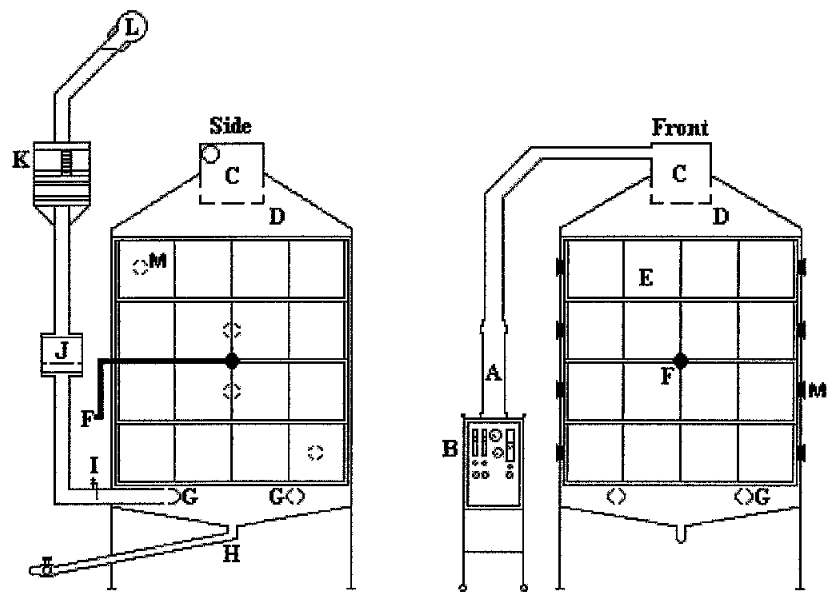


Key

1	Diluent air rotameters	a	Liquid from bulk source
2	Rotameter control valve	b	Test substance feed line filters
3	Reservoir pressure display	c	Liquid reservoir
4	Water bath	d	Load cell
5	Vapour generator	e	Nitrogen (pressure) inlet
6	Diluent air	f	Safety valve
7	Vapour/air mixture (to chamber)	g	Toggle valve
8	Load cell display	h	90 μm stainless steel filter
		i	Micro-metering needle valve
		j	Liquid line to vapour generator

FIGURE B

Schematic of an inhalation chamber used to expose rats



A	Vapour inlet pipe	G	Exhaust plenum
B	Air flow control and chamber monitoring	H	Drain
		I	Gate valve
C	Dispersion device	J	Pre-filter
D	Exposure chamber (0.75 m^3)	K	Powered extract filter
E	Animal exposure cages	L	Main exhaust
F	CEMS sampling port	M	Manual Sampling ports

TABLE A

Operating conditions for the rodent inhalation exposure system

Parameter	Group			
	1 (Air control)	2 (Low dose)	3 (Inter. dose)	4 (High dose)
Target concentration of 2-methyl-2-butene (ppm)	0	2000	4000	7000
Chamber airflows (l/min)				
Target air flow to vapour generator	N/A	30	90	150
Target supplementary airflow	150	120	60	N/A
Vapour generator settings				
Reservoir pressure (Helium, psi)	N/A	10	10	10
Sinter diameter (mm)	130	130	130	130
Chamber negative pressure (mm/H_2O)	4	4	4	4

N/A Not applicable

TABLE B

Chamber concentrations of 2-methyl-2-butene (ppm) - daily mean values

Exposure No.	Chamber Concentration (ppm)			
	Gp1 (Control	Gp2 (Low Dose)	Gp3 (Inter Dose)	Gp4 (high Dose)
1	BLQ	2142	3570	7361
2	BLQ	2057	4160	7237
3	BLQ	1865	4139	6394
4	BLQ	1894	4207	7571
5	BLQ	2002	4217	6977
6	BLQ	1294	3971	7457
7	BLQ	2376	4227	6907
8	BLQ	2138	3725	6966
Mean of Means	BLQ	1971	4027	7109
sd		317.4	251.3	378.8
CV		16.1	6.2	5.3

BLQ (LOQ) The peak detected was below the limit of quantification for the assay. The LOQ was set at 320 ppm (20% below the low tolerance level of the lowest calibration standard (nominally 400 ppm))

sd Standard deviation

CV Coefficient of variation (sd × 100/mean)

TABLE C

Nominal concentrations of 2-methyl-2-butene (ppm) - individual exposure values

Group 2 (Low dose) - Target concentration 2000 ppm

Exposure		Barometric pressure (mmHg)	2-methyl-2-butene usage (kg)	Chamber airflow[a] (l/min)	Chamber concentration		A/N ratio (%)
No.	Duration (min)				Nominal[b] (ppm)	Analysed (ppm)	
1	360	758	0.329	150	2099	2142	102
2	360	763	0.337	150	2136	2057	96
3	360	766	0.273	150	1723	1865	108
4	360	766	0.281	150	1773	1894	107
5	360	763	0.289	150	1831	2002	109
6	360	763	0.213	150	1351	1294	96
7	360	763	0.362	150	2295	2376	104
8	360	764	0.296	150	1876	2138	114
Mean of Means		763	0.298	150	1885	1971	105
sd		2.5	0.0460	0.0	293.8	317.4	6.3
CV		0.3	15.5	0.0	16.1	15.6	6.1

[a] Includes 30 l/min through vaporiser.

[b] Calculated from the following equations:

$$\text{Concentration (ppm)} = \frac{V}{V_a + V} \times 10^6$$

$$V = \frac{W \times R \times T}{M} \times \frac{760\ \text{mm Hg}}{\text{Atm}}$$

where
- V = gaseous volume of 2-methyl-2-butene (litres)
- W = mass of 2-methyl-2-butene (kg)
- M = molecular weight of 2-methyl-2-butene (70.14 g/mole)
- R = gas constant (0.08205 l atm mol^{-1} K^{-1})
- T = temperature (K), = temperature (°C, see Table D) + 273
- Atm = atmospheric pressure (mmHg)
- V_a = volume of air (litres) passing through the chamber during the exposure

A/N Analysed/nominal concentration ratio expressed as a percentage
sd Standard deviation
CV Coefficient of variation (sd × 100/mean)

TABLE C

(Nominal concentrations of 2-methyl-2-butene (ppm) - individual exposure values - continued)

Group 3 (Intermediate dose) - Target concentration 4000 ppm

Exposure No.	Exposure Duration (min)	Barometric pressure (mmHg)	2-methyl-2-butene usage (kg)	Chamber airflow[a] (l/min)	Chamber concentration Nominal[b] (ppm)	Chamber concentration Analysed (ppm)	A/N ratio (%)
1	360	758	0.64	150	4065	3570	88
2	360	763	0.73	150	4606	4160	90
3	360	766	0.71	150	4457	4139	93
4	360	766	0.72	150	4516	4207	93
5	360	763	0.73	150	4600	4217	92
6	360	763	0.65	150	4100	3971	97
7	360	763	0.73	150	4617	4227	92
8	360	764	0.65	150	4110	3725	91
Mean of Means		763	0.70	150	4418	4027	92
sd		2.5	0.041	0.0	222.4	251.3	2.6
CV		0.3	5.9	0.0	5.0	6.2	2.8

a Includes 90 l/min through vaporiser.

b Calculated from the following equations:

$$\text{Concentration (ppm)} = \frac{V}{V_a + V} \times 10^6$$

$$V = \frac{W \times R \times T}{M} \times \frac{760\,\text{mm Hg}}{\text{Atm}}$$

where

V	=	gaseous volume of 2-methyl-2-butene (litres)
W	=	mass of 2-methyl-2-butene (kg)
M	=	molecular weight of 2-methyl-2-butene (70.14 g/mole)
R	=	gas constant (0.08205 l atm mol^{-1} K^{-1})
T	=	temperature (K), = temperature (°C, see Table D) + 273
Atm	=	atmospheric pressure (mmHg)
V_a	=	volume of air (litres) passing through the chamber during the exposure

A/N Analysed/nominal concentration ratio expressed as a percentage

sd Standard deviation

CV Coefficient of variation (sd × 100/mean)

TABLE C

(Nominal concentrations of 2-methyl-2-butene (ppm) - individual exposure values - continued)

Group 4 (High dose) - Target concentration 7000 ppm

Exposure No.	Exposure Duration (min)	Barometric pressure (mmHg)	2-methyl-2-butene usage (kg)	Chamber airflow[a] (l/min)	Chamber concentration Nominal[b] (ppm)	Chamber concentration Analysed (ppm)	A/N ratio (%)
1	360	758	1.14	150	7238	7361	102
2	360	763	1.14	150	7196	7237	101
3	360	766	1.01	150	6354	6394	101
4	360	766	1.20	150	7540	7571	100
5	360	763	1.09	150	6878	6977	101
6	360	763	1.16	150	7321	7457	102
7	360	763	1.09	150	6915	6907	100
8	360	764	1.11	150	6999	6966	100
Mean of Means		763	1.12	150	7055	7109	101
sd		2.5	0.057	0.0	359.7	378.8	0.8
CV		0.3	5.1	0.0	5.1	5.3	0.8

[a] Includes 150 l/min through vaporiser.
[b] Calculated from the following equations:

$$\text{Concentration (ppm)} = \frac{V}{V_a + V} \times 10^6$$

$$V = \frac{W \times R \times T}{M} \times \frac{760\,\text{mm Hg}}{\text{Atm}}$$

where
- V = gaseous volume of 2-methyl-2-butene (litres)
- W = mass of 2-methyl-2-butene (kg)
- M = molecular weight of 2-methyl-2-butene (70.14 g/mole)
- R = gas constant (0.08205 l atm mol^{-1} K^{-1})
- T = temperature (K), = temperature (°C, see Table D) + 273
- Atm = atmospheric pressure (mmHg)
- V_a = volume of air (litres) passing through the chamber during the exposure

A/N Analysed/nominal concentration ratio expressed as a percentage
sd Standard deviation
CV Coefficient of variation (sd × 100/mean)

TABLE D

Chamber temperature and relative humidity - exposure mean values

Exposure No.	Mean chamber temperatures (°C) and relative humidity (%RH)							
	Group 1 (Air control)		Group 2 (Low dose)		Group 3 (Intermediate dose)		Group 4 (High dose)	
	Temp	RH	Temp	RH	Temp	RH	Temp	RH
1	21.9	37	21.2	42	20.5	36	21.3	39
2	21.9	36	21.2	43	20.6	28	21.5	32
3	21.5	36	21.0	46	20.2	32	21.4	30
4	21.4	32	20.9	41	20.0	30	21.4	29
5	21.5	33	21.0	45	20.2	28	21.3	32
6	21.8	31	21.1	44	20.4	30	21.5	33
7	20.4	32	21.3	38	21.3	30	22.9	31
8	20.9	28	21.5	42	21.5	25	21.5	29
Mean	21.4	33	21.2	42	20.6	30	21.6	32
sd	0.52	3.0	0.19	2.5	0.54	3.3	0.53	3.2
CV (%)	2.5	9.1	0.9	5.9	2.6	11.0	2.5	10.2

sd standard deviation
CV Coefficient of variation (sd × 100/mean)

APPENDIX A

Methods of sample collection and analysis for 2-methyl-2-butene

SAMPLE COLLECTION

Chamber concentration

Samples of chamber air were collected in sequence from each of the four exposure chambers (Chambers 4 - 1 sampled sequentially) and from one sampling point within each chamber. Air from each chamber was continually drawn through a transfer line, which was therefore equilibrated with the mean concentration from each chamber. When not being sampled, the air from the transfer lines was pumped to waste.

At approximately 7-minute intervals, the air from the transfer lines was switched to the injection loop of the Gas Chromatograph for automated analysis and data logging of the data.

METHOD OF ANALYSIS

Chamber atmosphere samples were analysed by gas chromatography. The method of sample analysis is detailed, together with a summary of the method validation, in the Inhalation Analytical Procedure at the end of this Appendix.

APPENDIX A

(Methods of sample collection and analysis for 2-methyl-2-butene - continued)

CALCULATIONS

GC analysis

The samples of chamber atmosphere were injected into a gas chromatograph, which was calibrated using vapour standards prepared in gas sampling bags. The method for calculating the concentration of 2-methyl-2-butene from the mass used to prepare each vapour standard is given below in equations 1 and 2.

$$\text{Concentration} = \frac{V}{Va + V} \times 1{,}000{,}000\ \text{ppm} \qquad (1)$$

$$V = \frac{W \times R \times T}{M} \times \frac{760}{Atm} \qquad (2)$$

where	V	=	gaseous volume of 2-methyl-2-butene (ml)
	W	=	mass of 2-methyl-2-butene (mg)
	M	=	molecular weight of 2-methyl-2-butene (70.14 g/mole)
	R	=	Gas constant (0.08205 ml atm $mmol^{-1}$ K^{-1})
	T	=	temperature (K)
	Atm	=	atmospheric pressure (mmHg)
	V_a	=	volume of air (ml)

APPENDIX A

(Methods of sample collection and analysis for 2-methyl-2-butene - continued)

Compound specific Inhalation Analytical Procedure for 2-Methyl-2-butene

The analysis of 2-methyl-2-butene in air

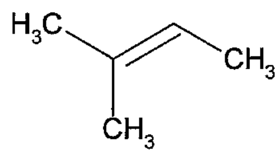
The method outlined in this document has been validated and is considered fit for the purpose of monitoring test atmospheres in an Inhalation Toxicology study.

This document details the basic procedures for the analysis of 2-methyl-2-butene sampled by automated gas valve from test atmospheres. The resulting samples, of approximate concentration 500 to 8500 ppm, are analysed by GC. Study specific amendments and additions will be detailed within a supplementary document.

EFFECTIVE DATE:	14 June 2001

Test substance

2-Methyl-2-butene, C_5H_{10}, molecular weight 70.14, has the following structure:



Appearance	Colourless liquid
Storage	4°C

APPENDIX A

(Methods of sample collection and analysis for 2-methyl-2-butene - continued)

Equipment

Balance and data printer	Sartorius	R160P with YDP-01
Syringes	Hamilton	500 series gas-tight (500 ml)
Gas sample bags	SKC INC	Tedlar® 232-series (10 and 20 dm^3 capacity)
Syringe valve	Mininert	Push button valve
Vacuum pump	AEG	ADEB 56 (or equivalent)
Flow meter	J & W Scientific	ADM1000 (acoustic displacement)
Wet gas meter	Zeal	DM3B (1 l)

Consumables

Gas bag tap septa	Sigma-Aldrich Chemical Co. Ltd	7.5 mm PTFE/Silicone septa
Syringes	Sigma Aldrich	20 ml polypropylene

APPENDIX A

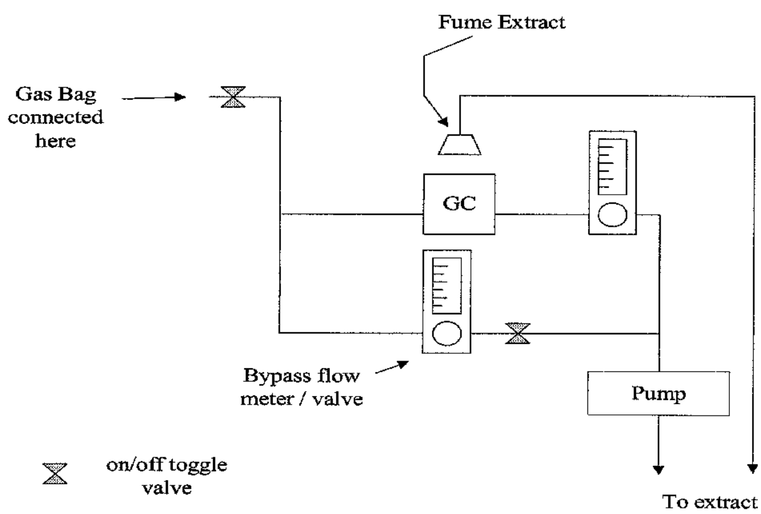
(Methods of sample collection and analysis for 2-methyl-2-butene - continued)

Preparation of samples for analysis

Gas Sample Analysis

Sample analysis by automated injection from gas bags is conducted by attaching each gas bag to the appropriate port (see Figure C). The pump is then switched on and immediately after, the gas bag tap is opened to allow the test compound to be drawn along the sample lines. For injections of the same concentration, the pump is switched on for a period of 60 seconds before starting the gas chromatograph and for dissimilar concentrations, 90 seconds is used to purge the sampling lines. The sampling flow valves are adjusted so that most (300 ml/min) of the chamber atmosphere passes through the bypass flow meter while only 60 ml/min passes through the GC injection valve (via 1/8" Teflon tubing).

Figure C - Automated Gasbag Sampling System



APPENDIX A

(Methods of sample collection and analysis for 2-methyl-2-butene - continued)

Preparation of calibration standards

Gas standards

Standards are prepared using the following method; the actual standard concentration ranges used are as detailed in the study specific supplement.

Collect the liquid sample directly from the storage cylinder into an empty glass vial fitted with a sealing valve. The glass vial is then stored at 4°C.

Evacuate gas sample bags of appropriate volumes and introduce measured volumes of air using a wet gas meter. Use a gas tight syringe fitted with a sealing valve to accurately dispense an aliquot of 2-methyl-2-butene into the top standard gas bag via the injection port. Using a gas tight syringe, accurately dispense measured volumes of the 2-methyl-2-butene vapour from the top standard gas bag into the gas sample bags via the injection port to produce standards covering the concentration range described in the study specific supplement.

Storage of standards and samples

The maximum storage periods for the various sample types are detailed below:

Sample type	Storage conditions	Storage period
Gas standards	Room temp., dark	7 days

APPENDIX A

(Methods of sample collection and analysis for 2-methyl-2-butene - continued)

Calibration and quantification

Gas analysis

Calibrate by injecting duplicates of each calibration standard solution, as detailed in the study specific supplement, at the beginning and end of each analytical sequence. Measure the peak area response in each injection of the calibration standard solutions and derive the line of best fit using a $1/\text{concentration}^2$ weighted least squares method.

For each injection of the sample measure the peak area response and determine the amount present in the sample using the equation below:

$$\text{Amount}(\mu g) = \frac{(A - I)}{S}$$

Where A = Peak area response of 2-methyl-2-butene in the sample chromatogram
S = Slope of calibration line derived from calibration data
I = Intercept of calibration line derived from calibration data

APPENDIX A

(Methods of sample collection and analysis for 2-methyl-2-butene - continued)

Chromatographic conditions

Analytical column	Chrompack CP-Sil 5CB, 5µ film, 30 m x 0.53 mm i.d.
Carrier gas	Helium (4.2 ml/min)
Split vent	Helium (48.3 ml/min)
Septum purge	Helium (1 ml/min)
Split ratio	1:12.5
Make up	Helium (48.3 ml/min)
Oxidant	Air (412 ml/min)
Fuel	Hydrogen (30 ml/min)
Injection volume	250 µl *via* an automated gas valve
Injector temperature	100°C
Detector temperature	250°C
Column temperature	Isothermal at 40°C for 5 minutes
Purge valve	Off at start of run. On at 0.01 min. Off at 1 min.
Retention time	2-Methyl-2-butene approximately 4.2 minutes

APPENDIX A

(Methods of sample collection and analysis for 2-methyl-2-butene - continued)

Quality assurance measures

Gas analysis

When the method is established on a chromatographic system six injections of a standard will be used to verify performance of the system. The parameters and acceptance criteria are set out below:

Parameter	Typical Value
Plate count (USP)	9760
Tailing factor (USP)	1.02
Repeatability (CV, n=6)	< 1.5%
QC tolerance	< ±5%
QC tolerance at LOQ	< ±10%

The highest calibration standard will be compared against a standard of similar concentration prepared independently. The ratio of response factors will be acceptable if within the range 0.95 to 1.05.

A quality check standard must follow every 6 concentration samples for the analysis to be regarded as valid. The results of the quality check standards must lie within the QC tolerance limits.

A quality check standard of low concentration will be run to verify the LOQ for the run. The LOQ for the run will be regarded as the concentration of the lowest acceptable quality check standard.

APPENDIX A

(Methods of sample collection and analysis for 2-methyl-2-butene - continued)

Summary of method validation

The raw data for the method validation is located in study CSS/001.

Comparison of test blanks, standards and test samples showed that the analyte was well resolved from any potential interfering peak.

Precision data showed coefficients of variation for 2-methyl-2-butene of less than 1% with solutions in the range of 8500 to 1300 ppm, increasing to 1.5% at 500 ppm.

Least squares regression analysis with a $1/\text{concentration}^2$ weighting of the peak area response against concentration of standard (500 to 8500 ppm) produced a correlation coefficient of 0.9999 and relative errors less than 2% in the range 8500 to 500 ppm. The Limit of Quantification (LOQ) for 2-methyl-2-butene will be set by the lowest acceptable check standard, however, the LOQ and Limit of Detection (LOD) are potentially as low as 65.88 and 21.74 ppm respectively (calculated statistically using the standard deviation obtained for a solution of concentration 500 ppm).

Standards of 2-methyl-2-butene in the range 500 to 8500 ppm stored at ambient temperature for 7 days and subsequently analysed against fresh standards showed concentrations within 5% of their nominal concentrations except at concentrations approaching 500ppm, the Limit of Quantification, where concentrations within 10% of their nominal were observed.

Intermediate precision data showed a difference of 1.3% in the mean result of the analysis of two batches of samples of 2-methyl-2-butene.

APPENDIX A

(Methods of sample collection and analysis for 2-methyl-2-butene - continued)

Chromatographs

System No.	Components of gas chromatography system		
	Manufacturer	**Model No.**	**Description**
1	Hewlett Packard	5890A	Chromatograph with capillary inlets, heated automatic gas sampling valve, ECD and FID
	Hewlett Packard	G1513A	Autoinjector }
	Hewlett Packard	18596CX	Controller }6890 Series Autosampler
	Hewlett Packard	G1512AX	Turntable }
	Thermo Finnegan[4]	SP4500	A/D interface
	Thermo Finnegan	PC1000	Integration software
2	Pye Unicam	PU4550	Chromatograph with gas valve and FID
	Pye Unicam	PU4700	Autosampler
	Thermo Finnegan	SP4500	A/D interface
	Thermo Finnegan	PC1000	Integration software
3	Shimadzu	GC-14A	Chromatograph with FID
	Shimadzu	AOC-1400	Autosampler
	Shimadzu	AOC-14	Autoinjector
	Shimadzu		Split injection system
	Thermo Finnegan	SP4500	A/D interface
	Thermo Finnegan	PC1000	Integration software
6	Shimadzu	GC-14A	Chromatograph with FID
	Shimadzu	MGS-4	Automated gas valve
	Shimadzu	SPL-14A	Split injection system
	Shimadzu	CR4-A	Integrator
7	Shimadzu	GC-14A	Chromatograph with FID
	Shimadzu	MGS-4	Automated gas valve
	Shimadzu	SPL-14A	Split injection system
	Shimadzu	CR4-A	Integrator
8	Hewlett Packard	5890A	Chromatograph with capillary inlets, heated automated gas sampling valve and FID
	Hewlett Packard	18593B	Autoinjector }
	Hewlett Packard	18596CX	Controller }7673 Autosampler
	Hewlett Packard	G1512AX	Turntable }
	Thermo Finnegan	SP4500	A/D interface
	Thermo Finnegan	PC1000	Integration software
9	Perkin Elmer	Autosystem XL	Automatic Chromatograph with programmable split/less capillary injector, heated automatic gas sampling valve and FID

ECD Electron capture detector
FID Flame ionisation detector

[4] Thermo Finnegan has previously traded as ThermoQuest, Thermo Separation Products (TSP) and Spectra Physics. Individual equipment items and manuals may be identified with these trade-names.

APPENDIX A

(Methods of sample collection and analysis for 2-methyl-2-butene - continued)

CSS/001 - STUDY SPECIFIC SUPPLEMENT to the Inhalation analytical procedure for 2-Methyl-2-butene

This supplement details additions and amendments to the procedure to be used for the GC assay of 2-methyl-2-butene obtained from air samples collected on the above study.

The assay, incorporating the additions and amendments, is suitable for the analysis of 2-methyl-2-butene at concentrations within the range of 500 to 8500 ppm.

Details given in this supplement supersede those in the compound specific IAP.

EFFECTIVE DATE :	**14 June 2001**

Analytical standard

Name	2-Methyl-2-butene
Batch number	KU00837KU
Purity	99.58%
Expiry date	None supplied
Supplier	Aldrich

Preparation of standard gasbags

Prepare standard gasbags in the nominal range 500 to 8500 ppm.

Calibration and Quantification

Calibration of the instrument is performed using 4 calibration standards covering the nominal range of approximately 500 to 8500 ppm.

Chromatographs

The analysis is performed using chromatograph 8.

APPENDIX A

(Methods of sample collection and analysis for 2-methyl-2-butene - continued)

EFFECTIVE DATE :	11 August 2001

Preparation of samples for analysis

Vapour samples are collected using an automated system fitted with electrically controlled valves, which are manipulated using the Chamber Environment Monitoring System (CEMS-2) software.

The test atmosphere is drawn directly from the inhalation chamber through the sample line to the gas-sampling valve located on the GC.

Initially, the gas-sampling valve of the GC is set to the "load" position and the valve is automatically switched to the "inject" position after 60 seconds. Simultaneously, the GC activates the start of the run sequence.

Preparation of samples for analysis

Vapour samples are collected using an automated system fitted with electrically controlled valves, which are manipulated using the Chamber Environment Monitoring System (CEMS-2) software.

The test atmosphere is drawn directly from the gas bag through the sample line to the gas-sampling valve located on the GC.

Initially, the gas-sampling valve of the GC is set to the "load" position and the valve is automatically switched to the "inject" position after 20 seconds. Simultaneously, the GC activates the start of the run sequence.

Chromatographs

The analysis is performed using chromatograph 6.

APPENDIX A

(Methods of sample collection and analysis for 2-methyl-2-butene - continued)

Chromatographic conditions

Carrier gas	Helium (2.1 ml/min)
Split ratio	1:25
Oxidant	Air (330 ml/min)
Fuel	Hydrogen (30 ml/min)
Injection volume	500 µl *via* an automated gas valve
Injector temperature	80°C
Detector temperature	250°C
Range	10^2
Column temperature	Isothermal at 30°C for 2.5 minutes
Gas Sample valve	Off at 2.5 mins
Retention time	2-Methyl-2-butene approximately 1.7 minutes

APPENDIX A

(Methods of sample collection and analysis for 2-methyl-2-butene - continued)

Storage of standards and samples

The storage conditions for the various sample types are detailed below:

Sample type	Storage conditions
Calibration standards	Temperature: At ambient (room) temperature except during analysis. Lighting: Normal lighting conditions (12 hours darkness, 12 hours illumination).

Calibration and quantification

Calibrate by injecting six replicates of each calibration standard gasbag. Measure the peak area response in each injection of the calibration standard and derive the line of best fit using an $1/\text{Concentration}^2$ weighted least squares method.

At the beginning of each analytical sequence, conduct a "Pre-Monitor Calibration check" to ensure the gasbags are within accepted tolerance limits of the calibration model.

Quality assurance measures

Run four QC standards covering the nominal range (including the LOQ) prior to the start of sample analysis.

Additional method validation for chromatograph 6

Precision data showed coefficients of variation for 2-methyl-2-butene of less than 0.5% with solutions in the range of 8500 to 500 ppm.

Least squares regression analysis with a $1/\text{concentration}^2$ weighting of the peak area response against concentration of standard (500 to 8500 ppm) produced a correlation coefficient of 0.99993 and relative errors less than 2.5% in the range 8500 to 500 ppm. The Limit of Quantification (LOQ) for 2-methyl-2-butene will be set by the lowest acceptable check standard, however, the LOQ and Limit of Detection (LOD) are potentially as low as 17.40 and 5.74 ppm respectively (calculated statistically using the standard deviation obtained for a solution of concentration 500 ppm).

A standard of 2-methyl-2-butene at a concentration of 8500 ppm stored at ambient temperature for 9 days and subsequently analysed against fresh standards showed a concentration within 5% of their nominal concentrations.

APPENDIX A

(Methods of sample collection and analysis for 2-methyl-2-butene - continued)

EFFECTIVE DATE :	28 August 2001

Preparation of standard gasbags

Prepare standard gasbag in the nominal range 12,000 ppm to quantify ASR values detected during the exposure.

APPENDIX B

Individual 2-methyl-2-butene concentration measurements

Exposure No.	Sample No.	Chamber Concentration (ppm)			
		Group 1 (Air control)	Group 2 (Low dose)	Group 3 (Inter. dose)	Group 4 (High dose)
1	1	BLQ	1704	1484	815
	2	BLQ	2108	2607	6656
	3	BLQ	2136	3842	7076
	4	BLQ	2138	3779	6645
	5	BLQ	2231	3764	10387 [2]
	6	BLQ	2220	3652	7380
	7	BLQ	2222	3628	7127
	8	BLQ	2241	3600	7106
	9	BLQ	2017	3611	7128
	10	BLQ	2066	3620	7258
	11	BLQ	2061	3587	7134
	12	BLQ	2128	3579	7075
	Mean [1]	BLQ	2142	3570	7361
	sd		77.2	331.4	1027.8
2	13	BLQ	1823	3704	868
	14	BLQ	2053	4642	7078
	15	BLQ	2032	4859	7052
	16	BLQ	2058	3564	7102
	17	BLQ	2076	4972	7267
	18	BLQ	2060	3955	7481
	19	BLQ	2045	3969	7357
	20	BLQ	2052	3882	7276
	21	BLQ	2055	4092	7256
	22	BLQ	2062	3929	7258
	23	BLQ	2061	3933	7268
	24	BLQ	2071	3960	7212
	Mean [1]	BLQ	2057	4160	7237
	sd		11.9	451.8	125.2

BLQ The peak detected was below the limit of quantification (LOQ) for the assay. The LOQ was set at 400 ppm (20% below the low tolerance level of the lowest calibration standard (nominally 500 ppm))

[1] The initial concentration measurement of each exposure was excluded from all calculations

[2] Concentration originally above standard range, value included in mean and standard deviation

sd Standard deviation

APPENDIX B

(Individual 2-methyl-2-butene concentration measurements - continued)

Exposure No.	Sample No.	Chamber Concentration (ppm)			
		Group 1 (Air control)	Group 2 (Low dose)	Group 3 (Inter. dose)	Group 4 (High dose)
3	25	BLQ	1917	3277	492
	26	BLQ	2050	4008	7210
	27	BLQ	2055	4018	7252
	28	BLQ	2018	4098	7366
	29	BLQ	2026	4169	7724
	30	BLQ	1951	4129	7378
	31	BLQ	1956	4109	3569
	32	BLQ	1540	4205	2110
	33	BLQ	1072	4180	4924
	34	BLQ	1900	4200	8472
	35	BLQ	1989	4192	7190
	36	BLQ	1962	4220	7139
	Mean [1]	BLQ	1865	4139	6394
	sd		299.4	73.7	1977.1
4	37	BLQ	1196	BLQ	BLQ
	38	BLQ	1900	4173	12085 [2]
	39	BLQ	1931	3770	6050
	40	BLQ	1920	4104	6767
	41	BLQ	1945	4317	7408
	42	BLQ	1932	4303	7379
	43	BLQ	1943	4272	7352
	44	BLQ	1948	4253	7305
	45	BLQ	1928	4267	7299
	46	BLQ	1924	4271	7249
	47	BLQ	1927	4277	7212
	48	BLQ	1542	4274	7178
	Mean [1]	BLQ	1894	4207	7571
	sd		117.8	157.2	1548.6

BLQ The peak detected was below the limit of quantification (LOQ) for the assay. The LOQ was set at 400 ppm (20% below the low tolerance level of the lowest calibration standard (nominally 500 ppm))

1 The initial concentration measurement of each exposure was excluded from all calculations

2 Concentration originally above standard range, value included in mean and standard deviation

sd Standard deviation

APPENDIX B

(Individual 2-methyl-2-butene concentration measurements - continued)

Exposure No.	Sample No.	Chamber Concentration (ppm)			
		Group 1 (Air control)	Group 2 (Low dose)	Group 3 (Inter. dose)	Group 4 (High dose)
5	49	BLQ	1869	3397	560
	50	BLQ	2295	4284	6852
	51	BLQ	2420	4261	6880
	52	BLQ	2336	4213	6876
	53	BLQ	2366	4233	6867
	54	BLQ	2353	4232	6815
	55	BLQ	2371	4250	6815
	56	BLQ	2335	4158	6876
	57	BLQ	2332	4217	7302
	58	BLQ	1153	4183	7200
	59	BLQ	830	4209	7164
	60	BLQ	1235	4147	7103
	Mean [1]	BLQ	2002	4217	6977
	sd		605.5	42.0	178.0
6	61	BLQ	1194	3301	616
	62	BLQ	895	3754	7262
	63	BLQ	1182	3748	7264
	64	BLQ	1592	3635	7230
	65	BLQ	1717	4260	6853
	66	BLQ	1719	3853	8242
	67	BLQ	1725	3878	7032
	68	BLQ	1708	3877	7715
	69	BLQ	BLQ[2]	3836	6639
	70	BLQ	1486	4583	9857
	71	BLQ	1050	4113	6953
	72	BLQ	971	4142	6981
	Mean [1]	BLQ	1294	3971	7457
	sd		489.3	277.0	909.1

BLQ The peak detected was below the limit of quantification (LOQ) for the assay. The LOQ was set at 400 ppm (20% below the low tolerance level of the lowest calibration standard (nominally 500 ppm))

1 The initial concentration measurement of each exposure was excluded from all calculations

2 For mean calculations, a value of 200 ppm (BLQ/2) has been used

sd Standard deviation

APPENDIX B

(Individual 2-methyl-2-butene concentration measurements - continued)

Exposure No.	Sample No.	Chamber Concentration (ppm)			
		Group 1 (Air control)	Group 2 (Low dose)	Group 3 (Inter. dose)	Group 4 (High dose)
7	73	BLQ	980	3587	489
	74	BLQ	2942	4398	7010
	75	BLQ	2735	4253	6996
	76	BLQ	2933	4246	6972
	77	BLQ	2743	4147	6953
	78	BLQ	2746	4198	6925
	79	BLQ	2725	4215	6879
	80	BLQ	2673	4228	6867
	81	BLQ	2796	4207	6871
	82	BLQ	647	4207	6824
	83	BLQ	1290	4214	6848
	84	BLQ	1902	4181	6835
	Mean [1]	BLQ	2376	4227	6907
	sd		762.3	63.9	66.6
8	85	BLQ	1517	3459	1350
	86	BLQ	1529	3647	5831
	87	BLQ	1575	3414	7154
	88	BLQ	1567	3431	7166
	89	BLQ	2810	3982	7094
	90	BLQ	2820	3780	7062
	91	BLQ	2221	3790	7270
	92	BLQ	2197	3779	6974
	93	BLQ	2197	3797	7038
	94	BLQ	2204	3791	7008
	95	BLQ	2184	3775	7022
	96	BLQ	2214	3786	7009
	Mean [1]	BLQ	2138	3725	6966
	sd		442.3	167.7	386.6

BLQ The peak detected was below the limit of quantification (LOQ) for the assay. The LOQ was set at 400 ppm (20% below the low tolerance level of the lowest calibration standard (nominally 500 ppm))

1 The initial concentration measurement of each exposure was excluded from all calculations

sd Standard deviation

PROTOCOL AND PROTOCOL AMENDMENTS

Study Number : **CSS/001**

CONFIDENTIAL

Huntingdon
Life Sciences

PROTOCOL

2-METHYL-2-BUTENE

DOSE RANGE FINDING STUDY IN RATS

BY INHALATION EXPOSURE

Sponsor

American Chemistry Council
1300 Wilson Boulevard
Arlington
VA 22201
USA

Research Laboratory

Huntingdon Life Sciences Ltd
Woolley Road
Alconbury
Huntingdon
Cambridgeshire
PE28 4HS
ENGLAND

Total number of pages: 17

Final Protocol

CONTACT DETAILS

Sponsor's Representative : E.J. Moran, pH.D., D.A.B.T.

Study Number : **CSS/001**

Huntingdon
Life Sciences

PROTOCOL APPROVAL

2-METHYL-2-BUTENE

DOSE RANGE FINDING STUDY IN RATS

BY INHALATION EXPOSURE

Amanda Brooker .... 19 June 2001

A.J. Brooker, B.Sc. (Hons.), M.Sc., C.Biol., M.I.Biol.
Study Director,
Huntingdon Life Sciences Ltd.

Date

The signature of the Study Director confirms this protocol as the working document for the study. Any changes made subsequent to the date of the Study Director's signature will be documented in formal amendments.

.... 19 June 2001

L.A. Waterson, B.Sc. (Hons.) I.D.T.
Management,
Huntingdon Life Sciences Ltd.

Date

.... July 12, 2001

E.J. Moran
Sponsor,
American Chemistry Council

Date

Please sign both copies of this page, retain one for your records and return one to the Study Director at Huntingdon Life Sciences.

Final Protocol

Study Number : **CSS/001**

Huntingdon Life Sciences

2-METHYL-2-BUTENE

DOSE RANGE FINDING STUDY IN RATS

BY INHALATION EXPOSURE

Enquiry Number: 21329L

Number of pages for internal distribution: 14

This working document is approved for circulation and use:

Amanda Brooker .... 19 June 2001
A.J. Brooker B.Sc (Hons.), M.Sc., C.Biol., M.I.Biol.
Study Director

Date

Primary location of study

Huntingdon Research Centre
Huntingdon
Cambridgeshire
PE28 4HS

Building Number: Y14, Room 011

All procedures to be performed at the above site.

Final Protocol

CONTENTS

1. INTRODUCTION

Management of study

Study Director	:	A.J. Brooker, B.Sc. (Hons.), M.Sc., C.Biol., M.I.Biol.
Monitoring Toxicologist	:	C.J. Hardy, B.Sc., Ph.D., M.I.Biol., C.Biol., Dip.R.C.Path (Toxicology) (Inhalation aspects)
		K. Hazleden, B.Sc., C.Biol., M.I.Biol. (Reproductive aspects)
In the temporary absence of the Study Director, the scientific responsibilities will be taken over by the Monitoring Toxicologist; other items of routine study management should be referred to the following person in the first instance.	:	D.W. Coombs, B.Sc.

Objective

Assessment of influence on mated female rats and the outcome of pregnancy, to establish suitable dosages for a 4-week General Toxicity and Reproductive/Developmental Toxicity Screening study.

Good Laboratory Practice

The work performed in this study will generally follow good laboratory practice principles, however, no specific study-related Quality Assurance procedures will be performed and the report may not contain all of the elements required by GLP.

Animals (Scientific Procedures) Act 1986 compliance

The in-life experimental procedures to be undertaken during the course of this study are subject to the provisions of the United Kingdom Animals (Scientific Procedures) Act 1986 (the Act). The Act, administered by the UK Home Office, regulates all scientific procedures in living animals which may cause pain, suffering, distress or lasting harm and provides for the designation of establishments where procedures may be undertaken, the licensing of trained individuals who perform the practical techniques and the issue of project licences for specified programmes of work.

This study will comply with all applicable sections of the Act and the associated Codes of Practice for the Housing and Care of Animals used in Scientific Procedures and the Humane Killing of Animals under Schedule 1 to the Act, issued under section 21 of the Act.

The number of animals used will be the minimum that is consistent with scientific integrity and regulatory acceptability, consideration having been given to the welfare of individual animals in terms of the number and extent of procedures to be carried out on each animal.

Animal model	:	Female CD rat (sexually mature, virgin), requirement for a rodent species by regulatory agencies, extensively used at this laboratory.
Route	:	Inhalation, to simulate the conditions of human exposure.

Treatment groups and dosages

Group	:	1	2	3	4
Compound	:	Control	------------- 2-methyl-2-butene -------------		
Dosage (ppm)	:	0	2000	4000	7000

Dosage levels were based upon available data for the components.

2. STUDY SCHEDULE AND STRUCTURE

2.1. Duration of treatment

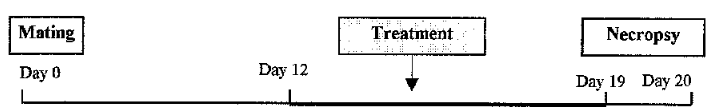
Females : Days 12 to 19 after mating.

2.2. Scheduled time plan

(to be up-dated as required in an amendment to protocol)

Sample of 2-methyl-2-butene to arrive	:	June 2001	
Animals to arrive	:	July 2001	
Experimental start (Treatment to commence)	:	July 2001	
Experimental termination date (Animal sacrifice)	:	August 2001	
Draft report to be issued	:	end September 2001	(estimated)

2.3. Study design



2.4. Identity of treatment groups

(to be selected from 26 animals ordered)

Group	Treatment	Dosage (ppm)#	Number of animals	Animal numbers
1	Control	0	6	1-6
2	2-methyl-2-butene	2000	6	7-12
3	2-methyl-2-butene	4000	6	13-18
4	2-methyl-2-butene	7000	6	19-24

Expressed in terms of the test substance as supplied (ppm).

3. TEST SUBSTANCE AND ADMINISTRATION

In order for Huntingdon Life Sciences to comply with the Health and Safety at Work etc. Act 1974, and the Control of Substances Hazardous to Health Regulations 1999, it is a condition of undertaking the study that the Supplier shall provide Huntingdon Life Sciences with all information available to it regarding known or potential hazards associated with the handling and use of any substance supplied by the Supplier to Huntingdon Life Sciences. The Supplier shall also comply with all current legislation and regulations concerning shipment of substances by road, rail, sea or air.

Such information in the form of a completed Huntingdon Life Sciences test substance data sheet must be received by Safety Management Services at Huntingdon Life Sciences before the test substance can be handled in the laboratory. At the discretion of Safety Management Services at Huntingdon Life Sciences, other documentation containing the equivalent information may be acceptable.

Information received will be used to set the Huntingdon Life Sciences Hazard Class, which determines safety precautions taken in the workplace.

Huntingdon Life Sciences Hazard Class:

3.1. Test substance

Sponsor's identification	:	2-methyl-2-butene.
CAS Number	:	To be advised.
Storage conditions	:	At ambient temperature or in a refrigerator, unless otherwise directed by the Sponsor. Any such decision will be documented in the study data and included in the final report.
Supplier's responsibilities	:	Documentation of methods of synthesis, fabrication or derivation. Stability data. Certificate of analysis.
Certificate of analysis details	:	Test substance identity. Batch number. Purity. Composition. Other appropriate characteristics. Current expiry date.

A retention sample as required under 40 CFR 792.105(d) will not be taken nor held for the period specified by 40 CFR 792.195 as the test substance is not indefinitely stable and may also pose a safety hazard. The identity, other characterisitics and stability under conditions at the test site shall be documented by appropriate analyses prior to study(s)start and completion.

3.2. Atmosphere generation

Equipment	:	All glass vaporiser.
Method	:	The test substance (liquid) will be metered to the vaporiser (through which air is passed) from a reservoir pressurised with inert gas. The air supply is provided from a compressor and is filtered, temperature and humidity controlled. The vapour/air mixture produced passes directly into the exposure chamber. The exposure system will be fully detailed in the report.
Concentrations	:	0, 2000, 4000, 7000 ppm.

3.3. Atmosphere sampling

Sampling	:	Samples of the test atmosphere from each exposure chamber will be drawn through a manifold by a diaphragm pump. At intervals, an automated valve system will divert a sample from each of the chambers in turn to a gas chromatograph. The samples will be injected onto a gas chromatograph (GC) by a motorised valve. The sampling frequency will be at least once per hour from each chamber of test atmosphere.
Concentration	:	The analysed concentration of significant components of the test atmospheres will be determined by capillary GC analysis with external standardisation. Spatial and temporal variations of concentrations in the chamber will be characterised during preliminary work without animals. Details of the analytical system will be confirmed by Protocol Amendment. The nominal concentration will be determined from the amount of test material used and the airflow through the chamber.

3.4. Control of test atmosphere characteristics

Pre-study system characterisation	:	Before commencement of treatment the system will be characterised at the target exposure vapour concentrations without animals in order to: • demonstrate reproducibility of vapour concentration. • demonstrate homogeneity of vapour concentration and distribution between levels in the chamber.

4. ANIMAL MANAGEMENT

4.1. Animals - supply, acclimatisation and allocation

4.1.1. Animals

Species	:	Rat.
Strain	:	Crl:CD® BR.
Age range ordered	:	Females 9-10 weeks of age.
Weight range ordered	:	Females 200-220 g.
Supplier	:	Females: Charles River (UK) Limited, Margate, Kent, England.
Special	:	Timed-mated by supplier, day of detection of positive mating = Day 0 of pregnancy.

4.1.2. Acclimatisation

Duration	:	Approximately 11 days before treatment commences.
Husbandry conditions	:	Refer to Section 4.2.

4.1.3. Allocation to treatment groups

Allocation	:	On arrival.
Method	:	Random.
Possible exceptions	:	Allocation adjusted if arrival group mean gestation bodyweights differ beyond acceptable limits.
Cage distribution	:	Arrangement designed to minimise environmental variables.

4.1.4. Identification

Numbering	:	Unique for each animal within study.
Method	:	Tail tattoo.
Cage labels	:	Uniquely identifying the occupants.

Huntingdon Life Sciences

4.1.5. Precommencement animal replacement

2 spare females will be ordered to replace any individuals rejected before the start of treatment.

Rejection before allocation	:	Ill health. Bodyweight extremes.
Replacement before treatment	:	All animals examined for general health and abnormalities on Day 11 of gestation. Any considered unsuitable may be replaced.
Replacement during treatment	:	None scheduled.

4.2. Animals - housing, diet and water supply

4.2.1. Environmental control

Rodent facility	:	Full barrier - to minimise entry of external biological and chemical agents.
Air supply	:	Filtered, not recirculated.
Temperature	:	Maintained within the range of 19-25°C.
Relative humidity	:	Maintained within the range of 40-70%.
Monitored continuously or daily.		Excursions outside these ranges documented in the study data.
Lighting	:	12 hours light : 12 hours dark.
Alarm systems	:	Activated on ventilation failure and when temperature/humidity limits exceeded.
Electricity supply	:	Public supply with automatic stand-by generators.

4.2.2. Animal accommodation

Non-exposure accommodation

Study period	Number of animals[+]		Cage material	Cage flooring
	Male	Female		
Gestation	-	Up to 3	Stainless steel	Stainless steel grid

+ Unless reduced by mortality or isolation.

Grid cages will be suspended above absorbent paper which will be changed at least twice each week. Cages, cage-trays, food hoppers and water bottles will be changed at appropriate intervals.

During exposure accommodation

The animals are transferred to separate individual cages in the exposure chamber during the 6 hour exposure period.

The individual animal exposure cages are of the suspended basket type, constructed of stainless steel mesh.

4.2.3. Diet and water supply

Copies of all certificates of analysis are stored in the archives, and copies will be included in the final report.

Diet supply

Diet name	:	UAR VRF1 Certified.
Diet type	:	Pelleted diet.
Availability	:	Non-restricted.
Certification	:	Before delivery each batch of diet is analysed by the supplier for various nutritional components and chemical and microbiological contaminants. Supplier's analytical certificates are scrutinised and approved before any batch of diet is released for use.

Water supply

Supply	:	Public drinking water.
Regulatory agency	:	U.K. Department of the Environment.
Availability	:	Non-restricted via polyethylene or polycarbonate bottles with sipper tubes.
Certification	:	Certificates of analysis are routinely received from the supplier.

4.2.4. Contaminants assay

It is the Sponsor's responsibility to advise Huntingdon Life Sciences of any specific contaminants likely to prejudice the outcome of the study. Analyses for such contaminants may be performed if requested by the Sponsor.

4.3. Animals - procedures

4.3.1. Administration

Route	:	Inhalation by whole-body exposure in 0.75 m^3 exposure chambers.
Chamber conditions	:	Chamber temperature, humidity and air flow will be monitored and recorded at intervals during exposure.
Treated at	:	Constant air borne concentration.
Controls (Group 1)	:	Air.
Frequency	:	Once daily for 6 hours per day, for 8 consecutive days (Days 12 to 19 of gestation).

4.3.2. Clinical observations

Animals and their cages	:	Visually inspected at least twice daily for evidence of reaction to treatment or ill-health.
Deviations from normal recorded at the time in respect of	:	Nature and severity. Date and time of onset. Duration and progress of the observed condition.

In addition detailed observations will be made on days of exposure according to the following frequency:

1. Pre-exposure observation.
2. Observation during exposure (restricted to gross changes on a group basis).
3. Within ½ to 1 hour of return to home cage.

The above schedule will be amended, as necessary, in the light of signs observed.

During the acclimatisation period observations of the animals and their cages will be recorded at least once per day.

4.3.3. Mortality

Premature sacrifice	:	Animals may be killed on humane grounds or if considered *in extremis.*
Animals found dead, killed *in extremis* or on humane grounds	:	A necropsy is performed as soon as possible. Animals found outside the normal workday will be preserved in a refrigerator (approximately 4°C) provided for this purpose.

4.3.4. Bodyweight

Females	:	Days 1, 3, 8, 12, 16 and 20 after mating.

4.3.5. Food consumption

Recorded	:	Days 1-2, 3-7, 8-11, 12-15, 16-19 after mating.

5. NECROPSY AND FETAL PROCESSING

5.1. Time of necropsy

Day 20 after mating.

5.2. Method of kill

All adult animals	:	Inhaled carbon dioxide.
Fetuses	:	Injection of sodium pentobarbital.

5.3. Macroscopic pathology

All adult females:

Macroscopic examination will be performed for evidence of disease or adverse reaction to treatment and abnormal tissues retained.

The number of corpora lutea in each ovary will be counted and the reproductive tract, complete with ovaries, will be dissected out. The following will be recorded:

Each ovary/uterine horn	:	Number of: Corpora lutea. Implantation sites. Resorption sites (early or late). Fetuses (live and dead). Fetuses and placentae dissected from the uterus and weighed individually. Fetuses sexed (external examination only).
Apparently non-pregnant animals	:	Status confirmed by Salewski staining technique for presence of implantation sites.
All fetuses	:	External examination only, then discarded.

5.4. Histology and light microscopy

(Optional)

Histological processing and microscopic examination of any retained tissues will only be performed, and documented in an amendment to the protocol, if requested by the Sponsor.

6. DATA TREATMENT

6.1. Data processing

Where appropriate group mean values with standard deviation (SD) will be calculated from individual data:

Presentation of data

Bodyweight of adult females	:	Group mean values and SD calculated from individual data. Gain over relevant periods may be calculated and graphically presented.
Food consumption of adult females	:	Group mean values and SD calculated for the periods specified in Section 4.3.
Reproductive tract, Day 20 after mating	:	Corpora lutea. Implantations. Resorptions (early, late, total). Viable young (male, female, total). Sex ratio (male:female offspring).
Pre-implantation loss	:	$\frac{\text{Number corpora lutea} - \text{Number implantations}}{\text{Number corpora lutea}} \times 100$
Post-implantation loss	:	$\frac{\text{Number implantations} - \text{Number viable fetuses}}{\text{Number implantations}} \times 100$
Litter weight	:	From individual litter weights (tabulated).
Fetal weights	:	Group mean values and SD calculated for male, female and overall from - $\frac{\text{Total of individual litter mean fetal weights}}{\text{Number of litters}}$
Placental weights	:	Group mean values and SD calculated from - $\frac{\text{Total of individual litter placental weights}}{\text{Number of litters}}$

6.2. Statistical analysis

The small sample size precludes meaningful statistical evaluation. Inter-group differences will be assessed by reference to the control data.

7. REPORTING

Study progress	:	Periodic verbal and written updates on study progress will be provided by the Study Director.
Draft final report	:	For review by Sponsor.
Authorised final report	:	After approval from the Sponsor.

Routinely reports are supplied on US Quarto paper. The following numbers of reports are supplied:

Type of report	Printing	Number of copies		
		Bound	Unbound	Electronic
Draft report	Single-sided	0	2	1
Authorised final	Double-sided Single-sided	1 0	0 1	1
Photographic report (if any)	Single-sided	1	0	

Any additions or corrections to an authorised final report will be documented as a formal addendum/amendment to the final report.

In the absence of ongoing communications, Huntingdon Life Sciences reserves the right to finalise, sign and issue the final report from this study six months after issue of the draft. In such an event, all materials will be transferred to the archive. Any subsequent requests for modifications, corrections or additions to the final report will be the subject of a formal report amendment (or new study, as appropriate) and will be subject to additional cost.

The data presented in the report will include but not be limited to the following:

Test substance information and analytical report

Strain, age, weight and source of animals used

Justification of administration route and rationale for dose level selection

Environmental conditions/animal husbandry and procedures

Certificates of analysis for diet and drinking water

Description of system and procedures for inhalation exposure

Results of the chamber concentrations

Clinical signs

Bodyweights

Food consumption

Litter measurements on Day 20 of gestation

Macroscopic pathology reports

Discussion/interpretation of results and conclusions

Robust summary

Study protocol and amendments

GLP compliance statement

8. QUALITY ASSURANCE AND ARCHIVING PROCEDURES

8.1. Quality assurance

No formal study-based Quality Assurance procedures will be performed on this study. These may be included if requested by the Sponsor – incorporated by protocol amendment.

8.2. Archiving

All raw data, samples and specimens arising from the performance of this study will remain the property of the Sponsor.

Types of sample and specimen which are unsuitable, by reason of instability, for long term retention and archiving may be disposed of after the periods stated in Huntingdon Life Sciences Standard Operating Procedures.

All other samples and specimens and all raw data will be retained by Huntingdon Life Sciences in its archive for a period of ten years from the date on which the Study Director signs the final report. After such time, the Sponsor will be contacted and his advice sought on the return, disposal or further retention of the materials. If requested, Huntingdon Life Sciences will continue to retain the materials subject to a reasonable fee being agreed with the Sponsor.

Huntingdon Life Sciences will retain the Quality Assurance records relevant to this study and a copy of the final report in its archive indefinitely.

Study Number : **CSS/001**
Protocol Amendment Number : **1**

Huntingdon
Life Sciences

2-METHYL-2-BUTENE

DOSE RANGE FINDING STUDY IN RATS

BY INHALATION EXPOSURE

Total number of pages: 3

Number of pages for internal distribution: 2

Study Director : A.J. Brooker, B.Sc. (Hons.), M.Sc., C.Biol., M.I.Biol.

The signature of the Study Director authorises the implementation of this amendment to protocol. In this amendment, deleted statements are struck through and new statements are underlined. Any changes to the study design after the date of this authorising signature will be documented in a further formal amendment.

AMENDMENT APPROVAL

For Huntingdon Life Sciences Ltd

Authorised by: Amanda Brooker Date: 27 July 2001
(Study Director)

For the Sponsor

Approved by: [signature] Date: Aug 15, 2001

Study Number : **CSS/001**
Protocol Amendment Number : **1**

Huntingdon
Life Sciences

2-METHYL-2-BUTENE

DOSE RANGE FINDING STUDY IN RATS

BY INHALATION EXPOSURE

Reasons for amendments	:	Updated time plan, and consequent change to recording days for data. The animals will arrive on Day 2 after mating. Correction to Sponsor's details

Amendments

2.2. Scheduled time plan

(to be up-dated as required in an amendment to protocol)

Sample of 2-methyl-2-butene to arrive	:	June 2001
Animals to arrive	:	~~July 2001~~ 10 August 2001
Experimental start (Treatment to commence)	:	~~July 2001~~ 20 August 2001
Experimental termination date (Animal sacrifice)	:	~~August 2001~~ 28 August 2001
Draft report to be issued	:	end ~~September~~ October 2001 (estimated)

4.3.4. Bodyweight

Females	:	Days ~~1, 3,~~ 2, 4, 8, 12, 16 and 20 after mating

4.3.5. Food consumption

Recorded	:	Days ~~1-2, 3-7,~~ 2-4, 5-7, 8-11, 12-15, 16-19 after mating

Study Number : **CSS/001**
Protocol Amendment Number : **1**

Huntingdon
Life Sciences

CONTACT DETAILS

Sponsor's Representative	: Elizabeth J. Moran, Ph.D., D.A.B.T. ~~E.J. Moran, pH.D., D.A.B.T.~~

CERTIFICATE OF ANALYSIS FOR RODENT DIET

ar

CONTROL DATA

VRF1CP2.5 lot 10213

Date of Manufacture 2001/02/13 Sell by date 2001/06/13
Use by date 2002/02/13

Bag numbers ..... :	301 à 350

Quantity manufactured .... *(tonnes)*	27
Variation from theoretical weight	*Conform*

SIEVE ANALYSIS (mm)

Diameter 0.00 - 0.10	0.2
Diameter 0.10 - 0.25	12.8
Diameter 0.25 - 0.50	44.3
Diameter 0.50 - 1.00	39.6
Diameter 1.00 - 2.00	3.1
Diameter 2.00 - 3.15	0.0
Diameter > 3.15	0.0

NUTRITIVE QUALITY

Incorporation of macro-mineral mix .... *(Na)*	Positive	
Incorporation of micro-mineral premix .... *(Mn and Cu)*	Positive	
Incorporation of vitamin premix .... *(Vit.A and E)*	Positive	
Moisture .... *(%)*	12.9	*(9 to 14)*
Crude protein .... *(%)*	18.5	*(17.4 to 20.4)*
Crude oil .... *(%)*	4.8	*(3.8 to 6.2)*
Nitrogen free extract .... *(%)*	54.4	*(48.0 to 60.0)*
of which starch .... *(%)*	40.4	
" which total sugars .... *(%)*	4.1	
Crude fibre .... *(%)*	3.6	*(2.8 to 5.2)*
Hemicellulose .... *(%)*		
True cellulose .... *(%)*		
Lignine .... *(%)*		
Total minerals .... *(%)*	5.8	*(4.5 to 7.0)*
Calcium .... *(mg / Kg)*	8 700	*(8 000 to 12 000)*
Phosphorus .... *(mg / Kg)*	7 200	*(4 000 to 8 300)*
Sodium .... *(mg / Kg)*	2 800	*(2 500 to 3 700)*
Potassium .... *(mg / Kg)*	8 100	*(5 700 to 9 700)*
Manganese .... *(mg / Kg)*	64	*(20 to 100)*
Copper .... *(mg / Kg)*	19	*(13 to 25)*
Vitamin A .... *(UI / Kg)*	43 100	*(20 000 to 55 000*
Vitamin C .... *(mg / Kg)*		
Vitamin E .... *(mg / Kg)*	100	

CONTAMINENTS

BACTERIOLOGY		
Viable organisms .... *(/g)*	15 000	*(< 100 000)*
Moulds and yeasts .... *(/g)*	< 10	*(< 1 000)*
Total coliforms .... *(/g)*	0	*(< 5)*
Faecal coliforms .... *(/g)*	0	*(0)*
Anaerobies S.R. .... *(/g)*	< 10	*(< 100)*
Salmonella ....*(/25 g)*	0	*(0)*

MYCOTOXINS *(µg / Kg)*		
Aflatoxin	< 1	*(< 5)*
Mycotoxin global risk	Negative	

Optional Notes

VRF1CP2.5 lot 10213 2001/02/13

HEAVY METALS

Lead - Pb .... (µg / Kg)	40	(< 1 500)
Mercury - Hg . (µg / Kg)	21	(< 100)
Arsenic - Ar . (µg / Kg)	10	(< 1 000)
Cadmium - Cd . (µg / Kg)	47	(< 250)
Selenium ..... (µg / Kg)	80	(< 600)

NITROGEN DERIVATIVES

NO2 .... (mg / Kg)	< 0.5	(Σ< 500)
NO3 .... (mg / Kg)	11.2	
NDMA .... (µg / Kg)	1.9	(< 10)
NDEA .... (µg / Kg)	< 0.2	(< 10)
NDPA .... (µg / Kg)	< 0.3	(< 10)
NDBA .... (µg / Kg)	< 0.3	(< 10)
NPIP .... (µg / Kg)	< 0.3	(< 10)
NPYR .... (µg / Kg)	< 0.5	(< 10)
NMOR .... (µg / Kg)	< 0.6	(< 10)

PESTICIDES ORGANOS-CHLORINE (µg / Kg) (Total < 200)

Lindane	< 1	(< 100)
a HCH	< 1	(< 20)
b HCH	< 5	(< 10)
d HCH	< 5	(< 100)
HCB	< 1	(< 10)
PCB	< 50	(< 50)
Aldrin	< 1	(< 10)
Dieldrin	< 1	(< 20)
Endosulfan	< 1	(< 100)
Heptachlor	< 1	(Σ< 10)
Heptachlor Epoxide	< 1	
Endrin	< 1	(< 10)
o.p'DDD	< 5	(Σ< 50)
p.p'DDD	< 5	
o.p'DDE	< 1	
p.p'DDE	< 1	
o.p'DDT	< 5	
p.p'DDT	< 5	

PESTICIDES ORGANOS-PHOSPHORUS (µg / Kg) (Total < 7 000)

Acephate	< 500	(< 5 000)
Azinphos ethyl	< 50	(< 5 000)
Azinphos methyl	< 50	(< 5 000)
Bromophos ethyl	< 10	(< 5 000)
Bromophos methyl	< 20	(< 5 000)
Carbophenothion ethyl .	< 50	(< 5 000)
Carbophenothion methyl	< 20	(< 5 000)
Chlorfenvinphos	< 10	(< 5 000)
Chlormephos	< 10	(< 5 000)
Chlorpyriphos ethyl ...	< 15	(< 5 000)
Chlorpyriphos methyl ..	< 15	(< 1 500)
Chlorthiofos	< 15	(< 5 000)
Diazinon	< 15	(< 5 000)
Dichlofenthion	< 10	(< 5 000)
Dichlorvos	< 20	(< 5 000)
Diethion	< 10	(< 5 000)
Dimefox	< 20	(< 5 000)
Dimethoate	< 30	(< 1 000)
Dioxathion	< 15	(< 5 000)
Disulfoton	< 30	(< 5 000)
Ethoprophos	< 20	(< 5 000)
Fenchlorphos	< 20	(< 5 000)
Fenitrothion	< 15	(< 5 000)
Fenthion	< 30	(< 5 000)
Fonofos	< 20	(< 5 000)
Formothion	< 20	(< 5 000)
Heptenophos	< 30	(< 5 000)
Iodofenphos	< 25	(< 5 000)
Malathion	39	(< 5 000)
Methamidophos	< 15	(< 5 000)
Methidathion	< 25	(< 5 000)
Mevinphos	< 10	(< 5 000)
Monocrotophos	< 90	(< 5 000)
Naled	< 15	(< 5 000)
Oxydemeton methyl	< 400	(< 5 000)
Parathion ethyl	< 20	(< 5 000)
Parathion methyl	< 20	(< 5 000)
Phosalone	< 50	(< 5 000)
Phosmet	< 50	(< 5 000)
Phosphamidon	< 25	(< 5 000)
Profenofos	< 50	(< 5 000)
Prothoate	< 20	(< 5 000)
Pyridaphenthion	< 15	(< 5 000)
Pyrimiphos ethyl	< 20	(< 5 000)
Pyrimiphos methyl	< 15	(< 2 500)
Sulfotep	< 20	(< 5 000)
Temephos	< 15	(< 5 000)
Tetrachlorvinphos	< 30	(< 5 000)
Thiomethon	< 40	(< 5 000)
Triazophos	< 30	(< 5 000)
Trichlorfon	< 10	(< 5 000)
Trichloronate	< 25	(< 5 000)

SYNTHETIC PYRETHRINOIDS (µg / Kg)

ND .... ND .... ND

NOTES

Laboratoire Contrôle AQ
Le Responsable

2001/05/10

Le Responsable AQ

CERTIFICATE OF ANALYSIS FOR DRINKING WATER

ANALYTICAL DATA SUMMARY SHEETS

Huntingdon North Public Water Supply Zone

Population:- 47616 | **01-Jan-01 - 30-Jun-01** | Zone Code:- FW40 | Grid Ref:- TL245735

Parameter		PCV	Units	Number of samples		% samples contravening PCV	Concentration or Value (all samples)		
Ref	Name						Minimum	Mean	Maximum
A001	Colour	20	mg/l Pt/Co	6	R	0	< 1	< 1.37	2.7
A002	Turbidity	4	FTU	34	R	0	0.08	< 0.173	0.57
A003	Odour	3	Dil No	5	R	0	< 0	< 0	< 0
A03a	Odour - Nature	-	-	34		-	1	1	1
A03b	Odour - Intensity	-	-	34		-	1	1	1
A004	Taste	3	Dil No	5	R	0	< 0	< 0	< 0
A04a	Taste - Nature	-	-	34		-	1	1	1
A04b	Taste - Intensity	-	-	34		-	1	1	1
A005	Temperature	25	°C	61		0	2.7	10.8	20.3
A006	Hydrogen ion (pH)	5.5 - 9.5	pH	34	R	0	7.58	7.75	8.13
A007	Sulphate	250	mg/l	1		0	113	113	113
A008	Magnesium	50	mg/l	1		0	7.94	7.94	7.94
A009	Sodium	150	mg/l	1		0	32.1	32.1	32.1
A09a	Sodium 80*	150	mg/l	3		0	0	48.6	0
A010	Potassium	12 (15)	mg/l	10	X	0	5.87	6.69	7.3
A011	Dry Residues	1500	mg/l	1		0	532	532	532
A012	Nitrate	50	mg/l	13		0	14.4	22.8	25.1
A013	Nitrite	0.1	mg/l	13	I	30.77	< 0.003	< 0.058	0.191
A014	Ammonium	0.5	mg/l	6		0	0.161	0.175	0.201
A017	Total organic carbon	-	mg/l	1		-	3.78	3.78	3.78
A020	Surfactants	200	µg/l	1		0	< 18	< 18	< 18
A021	Aluminium	200	µg/l	6	R	0	< 3	< 7.4	25.3
A022	Iron	200	µg/l	8	R	0	< 14	< 14	< 14
A023	Manganese	50	µg/l	6	R	0	< 1	< 1	< 1
A024	Copper	3000	µg/l	1	R	0	40.6	40.6	40.6
A025	Zinc	5000	µg/l	1	R	0	< 14	< 14	< 14
A026	Phosphorus	2200	µg/l	3		0	483	720	1050
A027	Fluoride	1500	µg/l	1		0	253	253	253
B003	Cyanide	50	µg/l	1		0	< 5.3	< 5.3	< 5.3
B007	Lead	50	µg/l	1	R	0	< 3.2	< 3.2	< 3.2
P014	Chlorotoluron	0.1	µg/l	6		0	< 0.01	< 0.01	< 0.01
P032	Diuron	0.1	µg/l	6		0	< 0.01	< 0.01	< 0.01
P048	Isoproturon	0.1	µg/l	6		0	< 0.01	< 0.01	< 0.01
P051	Linuron	0.1	µg/l	6		0	< 0.01	< 0.01	< 0.01
P113	Monuron	0.1	µg/l	6		0	< 0.01	< 0.01	< 0.01
P006	Bentazone	0.1	µg/l	6		0	< 0.01	< 0.012	< 0.02
P026	Dichloroprop	0.1	µg/l	6		0	< 0.01	< 0.012	< 0.02
P054	MCPA	0.1	µg/l	6		0	< 0.01	< 0.012	< 0.02
P053	MCPP(Mecoprop)	0.1	µg/l	6		0	< 0.01	< 0.012	< 0.02
P004	Atrazine	0.1	µg/l	10		0	< 0.01	< 0.01	< 0.01
P070	Prometryne	0.1	µg/l	10		0	< 0.01	< 0.01	< 0.01
P066	Propazine	0.1	µg/l	10		0	< 0.01	< 0.01	< 0.01
P073	Simazine	0.1	µg/l	10		0	< 0.01	< 0.011	0.02
P077	Terbutryne	0.1	µg/l	10		0	< 0.01	< 0.01	< 0.01
P132	Trietazine	0.1	µg/l	10		0	< 0.01	< 0.01	< 0.01
B010	Pesticides - Total	0.5	µg/l	10		0	0	0.006	0.02
B011	PAH	0.2	µg/l	1	R	0	0	0	0
C001	Total Coliforms	0	No/dl	63		0	0	0	0
C002	Faecal Coliforms	0	No/dl	63		0	0	0	0
C003	Faecal Streptococci	0	No/dl	2		0	0	0	0
C008	Colony Count 1Day @ 37°C	-	No/ml	63		-	0	0.905	8
C012	Colony Count 7Day @ 22°C	-	No/ml	63		-	0	43.1	308
C010	Chlorine Total	-	mg/l	61		-	0.15	0.609	0.95

ANALYTICAL DATA SUMMARY SHEETS

Huntingdon North Public Water Supply Zone

Population:- 47616 **01-Jan-01 - 30-Jun-01** Zone Code:- FW40 Grid Ref:- TL245735

Parameter Ref	Name	PCV	Units	Number of samples	% samples contravening PCV	Concentration or Value (all samples) Minimum	Mean	Maximum
D01a	Conductivity - M12	1500	µs/cm	34	0	741	752	767
D02a	Chloride - M12	400	mg/l	1	0	55	55	55
D03a	Calcium - M12	250	mg/l	1	0	128	128	128
D07a	Benzo 34 pyrene - M12	10	ng/l	1	0	3	3	3
D08a	Tetrachloromethane - M12	3	µg/l	6	0	0.078	0.089	0.1
D09a	Trichloroethene - M12	30	µg/l	6	0	0.308	0.355	0.4
D10a	Tetrachloroethene - M12	10	µg/l	6	0	0.235	0.268	0.3
E001	Hardness as Ca - Min	-	mg/l	1	-	158	158	158
E002	Alkalinity - HCO3 - Min	-	mg/l	1	-	253	253	253

Notes

PCV - Prescribed concentration or value
M12 - Rolling 12 month mean
M3 - Rolling 3 month mean
Min - PCV is a minimum only where the water is softened
U - Undertaking
X - Relaxation (relaxed value in brackets under PCV column)
R - Reduced sampling frequency
I - Increased sampling frequency
PAH - Polycyclic aromatic hydrocarbons

Sodium 80* - the 80th percentile of the last 3 years of sodium results

HUNTINGDON RESEARCH CENTRE GLP COMPLIANCE STATEMENT 2001

DEPARTMENT OF HEALTH

THE DEPARTMENT OF HEALTH OF THE GOVERNMENT OF THE UNITED KINGDOM

GOOD LABORATORY PRACTICE

STATEMENT OF COMPLIANCE IN ACCORDANCE WITH DIRECTIVE 88/320 EEC

LABORATORY	TEST TYPE
Huntingdon Life Sciences **Huntingdon Research Centre** **Wooley Road** **Alconbury** **Huntingdon** **Cambs.** **PE28 4HS**	**Analytical Chemistry** **Clinical Chemistry** **Ecosystems** **Environmental Fate** **Environmental Toxicity** **Phys/Chem Testing** **Toxicology**

DATE OF INSPECTION
15th January 2001

A general inspection for compliance with the Principles of Good Laboratory Practice was carried out at the above laboratory as part of UK GLP Compliance Programme.

At the time of the inspection no deviations were found of sufficient magnitude to affect the validity of non-clinical studies performed at these facilities.

Roger G. Alexander
3/4/01

Dr. Roger G. Alexander
Head, UK GLP Monitoring Authority